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Hindrance of Coliform Recovery by Turbidity and Non-Coliforms

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16. ABSTRACT

The objectives of this project were to evaluate the recoverability of coliforms from waters which have: a) high populations of non-coliform organisms, and b) high levels of turbidity due to natural mineral turbidity, hydrated oxides and organic debris. After initial isolation and identification of coliforms and non-coliforms from raw and distribution water interactions between these two groups of organisms were studied. The outcome of the interaction was found to be dependent upon numerous factors. These included: the specific non-coliform, the numbers of each type of organism, the nutrient environment in which the interaction occurred, the physiological status of the interacting organisms and the type of media used to recover the coliforms.

Turbidity augmentation and reduction experiments were done to distinguish non-bacterial turbidity inhibition of coliforms from the inhibition caused by other bacteria. The more serious inhibition to coliform detection seemed to be caused by the large populations of non-coliforms which exceeded the resident coliforms in water mains by factors of 10^2 to 10^5. Turbidity per se was not an impediment to coliform growth, but it did make it more difficult to recognize coliforms on membrane filters. These results will be of interest to individuals concerned with water quality and interpretation of microbiological data.

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FOREWORD

The Environmental Protection Agency was created because of increasing public and government concern about the dangers of pollution to the health and welfare of the American people. Noxious air, foul water, and spoiled land are tragic testiony to the deterioration of our natural environment. The complexity of that environment and the interplay between its components require a concentrated and integrated attack on the problem.

Research and development is that necessary first step in problem solution and it involves defining the problem, measuring its impact, and searching for solutions. The Municipal Environmental Research Laboratory develops new and improved technology and systems for the prevention, treatment, and management of wastewater and solid and hazardous waste pollutant discharges from municipal and community sources, for the preservation and treatment of public drinking water supplies, and to minimize the adverse economic, social, health, and aesthetic effects of pollution. This publication is one of the products of that research; a most vital communications link between the researcher and the user community.

This paper describes studies done on factors which affect interactions between coliforms and non-coliforms isolated from the distribution system. Additional studies were done on the effect of non-bacterial turbidity on coliform recovery. The data obtained provides information to all investigators who are concerned with water quality and who are responsible for interpretation of microbiological data.

Francis T. Mayo, Director
Municipal Environmental Research
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ABSTRACT

The principal objectives of this project were to evaluate the recoverability of coliforms from waters which have a) high populations of non-coliform organisms and b) high levels of turbidity due to natural mineral turbidity, hydrated oxides and organic debris.

The approach to the first objective was to isolate and identify coliform and non-coliform organisms from Brandywine River source water and from the Wilmington Water Department distribution system. Sampling was done at various times of the year to get as wide a range of organisms as possible.

After initial isolation and identification of coliforms and non-coliforms, our studies concentrated on the interactions between these two groups of organisms. When interactions between E. coli, Flavobacterium sp., Acinetobacter sp., Arthrobacter sp. and a CDC group II K-1 sp. were studied all organisms except for the latter one were found to be capable of inhibiting the coliform. The greater the numbers of non-coliforms the more pronounced the inhibition. The medium in which the interaction was studied also was important. The standard growth medium used was a low nutrient bacterial extract produced from organisms originally isolated from dead end water. Supplementation of this extract with a salts mix and phosphate buffer appeared to protect the coliforms from inhibition by the non-coliforms. Supplementation with washed tubercle solids appeared to have no effect.

Another important factor in determining the outcome of an interaction was the physiological status of both members. Coliform inhibition was not observed unless the non-coliform was able to grow and increase in numbers in the growth medium. This was also true for the coliform member of the interaction. In those studies where the coliform control numbers did not increase, but were only maintained in the eight day course of the experiment, inhibition of the coliforms by the non-coliforms was no longer observed in the coliform: non-coliform mix. Therefore coliforms may be less vulnerable to inhibition or stress by non-coliform when either member of the interaction is not actively growing. However, when inhibition is occurring, preliminary data indicates that the coliforms are being stressed by the non-coliforms. Under these conditions recovery on selective media is less than recovery on non-selective media.

A series of experiments, some with raw water and some with distribution system water, were set up to distinguish turbidity inhibition of coliforms from the inhibition caused by other bacteria. It was found that within those turbidity limits acceptable to the consumer, turbidity per se was not an impediment to coliform growth, but it did make it more

difficult to recognize coliforms on membrane filters. The strikingly persistent tendency for larger sample volumes on membrane filters to give proportionately smaller coliform yields is not attributable to turbidity. The more serious inhibitions to coliform detection seemed to be caused by surprisingly large populations of non-coliforms exceeding the resident coliforms in water mains by factors of 10^2 to 10^5.

This report was submitted in fulfillment of Grant #R805102 by the University of Delaware under the sponsorship of the Drinking Water Research Division, MERL, U. S. Environmental Protection Agency. This report covers the period March 13, 1977 to August 15, 1979, when work was completed.

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LIST OF ABBREVIATIONS AND SYMBOLS

BGB	-- Brilliant Green Bile Broth
LES-Endo	-- An Endo agar formulated for membrane filter work
LS	-- Lauryl sulphate (Lauryl Tryptose) broth
M-Endo Broth	-- A broth designed for membrane filter work
MF	-- Membrane filter or membrane filter method
M-5	-- A low nutrient, high iron, plate count agar
MPN	-- Most Probable Number technique, usually used with LS broth in coliform enumeration
PCA	-- Plate Count Agar
VRBA	-- Violet Red Bile Agar

SECTION 1

INTRODUCTION

One coliform per 100 ml of water is the standard currently in use for drinking water. Situations have been known to exist in the water distribution system of the Wilmington Water Department where 1-2 coliforms per 100 ml had to be isolated from water containing a total of 100 to 100,000 total organisms per ml. Attempts were made to approximate these as well as other conditions in the distribution system in our study of coliform:non-coliform interactions. Standard procedures for these studies were established using organisms isolated from the distribution system growing in a bacterial extract prepared from dead end water isolates. The experiments reported attempted to determine the effects of:

1. the specific non-coliform
2. the ratio of coliform:non-coliform
3. the media used
4. the physiological status of each member on the coliform: non-coliform interaction.

In addition, coliforms must be detected in waters containing high levels of turbidity due not only to high bacterial numbers but also to natural mineral turbidity, hydrated oxides, and organic debris. This situation, which is not unique to the Wilmington Water Department, serves to complicate detection of coliforms by routine analysis. Natural mineral turbidity, hydrated iron oxides, organic debris, and the live cells of other bacteria can all form mats on the MF surface from as little as 100 ml of a water sample. Their presence may inhibit coliforms, render the Endo components less inhibitory toward non-coliforms or promote confluent growth. In addition, if the turbidity is due to particles with embedded bacteria, these bacteria may be protected from the effects of chlorination. Inhibition of coliforms by non-coliforms can potentially pose a serious health hazard if any of the non-coliforms are pathogenic.(1)

In the turbidity studies the MF and MPN techniques were employed for coliform detection. Both methods were used side by side wherever possible. This was because it was assumed that the MF technique would be vulnerable to turbidimetric interference and that the MPN technique would be almost immune to such interference, but extremely vulnerable to non-coliform bacterial interference.

Most of the turbidity experiments were carried out in water from the distribution system frequently blended from several districts, and some-times containing water from a 2 meter cross section of 150 mm main cut from a neighborhood experiencing coliform regrowth problems. This

isolated main was kept active for many months by circulating small amounts of dead-end water through it with a peristaltic pump.

The experiments reported may be divided into 2 groups:

(1) Turbidity augmentation experiments where coliforms and the general bacterial population were measured before and after the addition of a chlorine-free main deposit. This adds a turbidity which covers a broad spectrum, as it was derived from water mains by way of hydrants on 150 mm branches.

(2) Turbidity reduction experiments where coliforms and total bacteria were measured before and after a low speed centrifugation designed to remove most of the turbidity while leaving most of the bacteria in the supernatant. The centrifuging procedure subtracted the largest and heaviest particles so that the residual turbidity was probably more organic, smaller in particle size, and lower in specific gravity than the original turbidity. Use of both of these techniques subjected standard coliform detection procedures to a wide variety of turbidimetric interference.

A determination of the effects of the above factors on coliform recovery would provide important information to all investigators who are concerned with water quality and who are responsible for interpretation of microbiological data. Ultimately this information may be used to provide further insights into alternate methods of water treatment which would diminish or eliminate the high numbers of microbes and high levels of turbidity. It may also be used to develop techniques to enable the detection of coliforms in water containing high numbers of non-coliforms and/or high turbidities so that this water is not erroneously thought to be safe.

SECTION 2

CONCLUSIONS

A wide variety of coliform and non-coliform organisms were isolated from Brandywine River water and from the Wilmington Water Department distribution system. Four of these isolates (*Flavobacterium* sp., *Acinetobacter* sp., *Arthrobacter* sp. and a CDC Group II K-1 sp.) were tested in interaction experiments for their ability to inhibit *E. coli*. The coliform was inhibited by all the non-coliforms except for the CDC Group II K-1 isolate. Inhibition of the coliform was observed to be dependent upon a number of factors. When the coliform: non-coliform ratio was decreased from 1:1 to 1:10 the inhibition was greater as determined by the recoverability of coliform colonies on plate count agar. The medium in which the interaction was studied was also important. Dead end water from the distribution system was found to be unsatisfactory as a growth medium because of the inability to have a coliform control without altering the water in the process of ridding it of the non-coliform indigenous population. The growth medium used was a low nutrient bacterial extract produced from organisms originally isolated from dead end water. Supplementation of this extract with a salts mix and phosphate buffer appeared to protect the coliforms from inhibition by the non-coliforms. Supplementation with washed tubercle solids appeared to have no effect on the coliform:non-coliform interaction studied.

Another important factor in determining the outcome of a coliform: non-coliform interaction is the physiological status of both members. This is demonstrated by comparing the growth of the coliform and non-coliform controls (each species growing alone in the test medium) with the growth of each of the two species when they are mixed together in the same medium. Coliform inhibition is not observed unless the non-coliform control is able to grow and increase in numbers in the growth medium. This is also true for the coliform member of the interaction. In the studies where control coliform numbers are not observed to increase, but are only maintained in the eight day course of the experiment, inhibition of the coliform by the non-coliform is no longer observed in the coliform: non-coliform mix. Therefore it appears that coliforms may be less vulnerable to inhibition or stress by non-coliforms when either member of the interaction is not actively growing. However, when inhibition is occurring preliminary data indicates that the coliforms are being stressed by the non-coliforms. Under these conditions recovery on selective media is less than recovery on non-selective media.

Turbidity *per se* is not the major hindrance to the growth of coliforms on membrane filters. On the other hand it does produce colonies more difficult to recognize as coliforms. With most of the waters

studied, proportionately larger quantities of water filtered through membrane filters do not give proportionately higher yields of coliform colonies. This phenomenon is still observed when the turbidity of a water sample is reduced by low speed centrifugation which does not remove a high proportion of the bacterial density. It appears then that the inhibition of coliforms is due more to the presence of large numbers of non-coliform bacteria rather than to the suspended matter present. The intercolonial spaces on incubated MF Endo membrane filters contain coliforms which have been inhibited and have not produced discernible coliform colonies. The inhibitory influence of non-coliforms upon coliform detection has probably escaped notice because the total bacterial population has not been measured. The M-5 agar medium used in one turbidity study gives very high colony yields, but other low nutrient media may serve as well.

SECTION 3

RECOMMENDATIONS

Interactions between coliforms and non-coliforms can be expected to occur in the distribution system. The outcome of the interaction is dependent upon numerous factors including the specific non-coliform present, the numbers of each type of organism, the nutrient environment in which the interaction is occurring, the physiological status of the interacting organisms and the type of media used to recover the coliforms. It is therefore important that investigators testing for the presence of the indicator coliform organisms be aware of these factors and their influence on coliform recovery.

Additional studies on the coliform: non-coliform interaction should be done. Our preliminary data indicates that when grown in the presence of non-coliforms, the coliforms may be stressed. If this is so, the ability to recover them using some standard techniques will be diminished. In addition, waters containing high numbers of non-coliforms in the absence of coliforms may not be considered safe until further studies are done to eliminate the possibility of the presence of stressed coliforms.

Purveyors of drinking water should concentrate their bacterial monitoring (efforts) in the chlorine-free or low chlorine outskirts of distribution systems rather than in the fresh output of wells and water treatment plants.

Wherever turbidity is such that the filtration of 100 ml leaves a conspicuous deposit on a membrane filter, the MPN method should be used.

If particular sampling points consistently yield heavily turbid cultures in the MPN test, transfers should be made to BGB even though gas is not present at the presumptive stage.

Where chlorine is low or absent a long term low nutrient plate count should be employed to determine the size of the background bacterial population within which coliform enumeration is being attempted.

SECTION 4

EXPERIMENTAL PROCEDURES

ISOLATION OF ORGANISMS

Organisms were isolated by the Wilmington Water Department and identified at the University laboratory. Primary media for coliforms included Lauryl Tryptose, Mac Conkey, Eosin Methylene Blue and Desoxycholate Agar. Plate count agar and M-5 agar were used for non-coliforms. All of the organisms were routinely Gram stained and tested in the API 20E system. The definition of coliform used here is the same as that in Standard Methods, 14th ed. (2).

In those cases where the API did not yield a number usable for identification of an isolate, Bergey's Manual (3) was used as a guide to determine the genus and if possible, species. Supplementary biochemical tests were done using standard procedures from the Manual for Clinical Microbiology (4) and Diagnostic Microbiology (5). In addition, American Type Culture Collection samples were used to further confirm our identifications. Tables 1 and 2 list the identity of the isolated organisms according to Geldreich et al.'s classification of total coliforms, coliform antagonists, opportunistic pathogens and category not established (6) and according to von Graevenitz's classification of opportunistic pathogens (7).

TABLE 1. ORGANISMS ISOLATED FROM RAW WATER

Category	Organism name	API
1. Total coliforms	Enterobacter cloacae	1305573
		3305723
		3305573
		3305773
		3305763
		1305173
	Citrobacter freundii	3704553
		1604532
	Enterobacter agglomerans	1205573
		1004353
		1244573
		1297573
	Escherichia coli	5144572
		1144573
		1144572
		7145552
	Klebsiella pneumoniae	1205773

(Continued)

TABLE 1. (Continued)

* Interaction studies with these organisms described in this report indicate they are also coliform antagonists.

** These organisms are considered opportunistic pathogens by von Graevenitz (7), but were placed in category not established by Geldreich et al. (6).

TABLE 2. ORGANISMS ISOLATED FROM FINISHED WATER

Category	Organism name	API
1. Total coliforms	Enterobacter cloacae	3305533
		3305773
		3305573
		3205533
	Citrobacter freundii	3704553
		1344573
	Enterobacter agglomerans	1205573
		1005573
	Escherichia coli	1144572
	Klebsiella pneumoniae	5255773
		5215773
2. Coliform antagonists	Pseudomonas maltophilia	100000040
	Pseudomonas sp. 2	220000451
		000200451
	Flavobacterium sp.	0 API
	Bacillus sp.	2202000
	*Acinetobacter sp.	11051735
		0204040
		33051531
		0 API
		4200000
		1000000
	*Arthrobacter sp.	1003000
		0001000
		0002000
		1002000
3. Opportunistic pathogens		
	Pseudomonas maltophilia	100000040
	Klebsiella pneumoniae	5255773
		5215773
	Serratia liquifasciens	53077635
	Moraxella sp.	000000440
		000100400
		2000000
	Aeromonas hydrophilia	70071075
		3047526
	**Bacillus sp.	2202000
	**Corynebacterium sp.	00000004
		004300004

(Continued)

TABLE 1. (Continued)

Category	Organism name	API
	Klebsiella pneumoniae	5255773
		5215773
		5255763
2. Coliform antagonists	Pseudomonas maltophilia	020000040
		020200040
	Pseudomonas aeruginosa	2202000
	Pseudomonas sp. 2	220000451
	Flavobacterium sp.	000000040
		000200010
	Bacillus sp.	30071234
		22020044
		02030000
		0202000
	*Acinetobacter sp.	0201000
		100717357
		0 API
		2204000
	*Arthrobacter sp.	0002000
		1203000
		040200400
	Actinomycetes	0 API
3. Opportunistic pathogens		
	Pseudomonas aeruginosa	2202000
	Pseudomonas maltophilia	020000040
		020200040
	Klebsiella pneumoniae	1205773
		5255773
		5215773
		5255763
	Acinetobacter calcoaceticus	0201000
	Aeromonas hydrophilia	70471245
		33471775
		30461045
	**Bacillus sp.	30071234
		22020044
		020300000
		0202000
		0201000
	**Corynebacterium sp.	0002004
	Flavobacterium sp.	000000040
		000200010
4. Category not established	Streptomyces sp.	1002000
		1002000
		1003000
		0203000

(Continued)

TABLE 2. ORGANISMS ISOLATED FROM FINISHED WATER

Category		Organism name	API
	Opportunistic pathogens	Flavobacterium sp.	0 API
4.	Category not established	CDC Group II K-1	000000442
		Yersinia pseudotuberculosis	1014153

* Interaction studies with these organisms described in this report indicate they are also coliform antagonists.

** These organisms are considered opportunistic pathogens by von Graevenitz (7), but were placed in category not established by Geldreich et al. (6).

Analysis of the isolated organisms indicates that the same species are found in finished and raw waters. Numerous organisms categorized as opportunistic pathogens are found in both waters. As noted on the tables our interaction studies indicate that Acinetobacter sp. and Arthrobacter sp. can be added to the list of coliform antagonists. In agreement with Geldreich et al. (6) we found Flavobacterium sp. also to be a potential coliform antagonist.

INTERACTION EXPERIMENTS

In our study of interactions between coliforms and non-coliforms standard conditions had to be established. Each of the following was considered:

Organisms to be Used

The organisms used in the study were isolates from the Wilmington Water Department distribution system. Their identification is indicated in the previous section.

Determination of Numbers of Coliforms and Non-Coliforms

For those studies where coliform and non-coliform numbers were equal or relatively close, Plate Count Agar (PCA), was used to determine total numbers of bacteria and Endo Agar was used to enumerate coliforms. When coliforms were grown alone, their numbers on PCA correlated well with their numbers on Endo Agar.

Growth Medium

An organism's growth characteristics vary depending on the type and concentration of medium used. It is expected that their interaction with other organisms will vary accordingly. Since the distribution system is a low nutrient environment we attempted to duplicate this condition in our experiments.

Dilute Nutrient Broth--

Concentrations of 2×10^{-3} and 1×10^{-3} nutrient broth were used in preliminary interaction screening experiments. Use of this medium was discontinued because it did not qualitatively or quantitatively duplicate the water environment.

Dead end Water From the Wilmington Water Department Distribution System--

This water was chosen because:

1. It is from the distribution system.
2. It supports the growth of microbes.
3. It may be that in this water coliform recovery is hindered by the presence of non-coliforms.

The major problem with using this water untreated is that it is impossible to have a proper control for coliform growth alone. The untreated (i.e., not filtered or autoclaved) water has microbes present which may affect coliform growth or recovery. Procedures used to remove these indigenous organisms would also alter the water chemically or physically. The growth of the coliforms alone in dead end water in the absence of the other organisms but with everything else present can therefore not be accurately assayed. We felt that under these conditions a way to estimate coliform control numbers would be to determine the growth of these organisms in untreated dead end water and in autoclaved dead end water.

As a preliminary experiment we found these studies to be very useful, but decided against continuing the use of dead end water because of the problem noted above with the coliform control. In all cases coliforms were undetectable by MPN assays by the sixth day of the experiment. We did not know if this was due to the inability of the water to support the growth of these organisms or if it was due to the inhibitory action of other organisms. The autoclaved water control was also not able to support the growth of coliforms. However, this may have been because of the effect of autoclaving which may have destroyed some organic material and/or produced toxic materials.

Bacterial Extract--

Our studies using dead end water as a growth medium led us to conclude that problems with setting up a proper coliform control far outweighed the advantages of using dead end water. We felt that in addition to the above noted problem, the dead end water would most likely vary chemically from time to time during the course of a year. We therefore decided to prepare a medium in which to grow the organisms.

Since lysed bacterial cells may themselves be providing nutrients for other organisms in the distribution system, (8) we decided to prepare bacterial extracts and test the ability of E. coli (C 29) and a non-coliform mix made up of organisms originally isolated from the distribution system to grow in these extracts.

Preparation of bacterial extracts--

A. Early (log phase) non-coliform extract

(1) Seven non-coliform organisms were isolated from the dead end water tested in the previous experiment. They were identified as:
1 Moraxella sp., 1 Flavobacterium sp., 3 Arthrobacter sp., 1 Staphylococcus saprophyticus, and 1 Streptomyces sp.

(2) All the organisms were grown on PCA plates, colonies were scraped and used to inoculate 1 liter of nutrient broth in 2 liter flasks. The flasks were allowed to shake at room temperature for 9 hours at which time the cultures were in logarithmic phase.

(3) Cells were harvested in 150 ml buckets spun at 10,000 RPM for 15 min. The cell preparations were kept on ice for the remainder of the procedure.

(4) The cell pellet was resuspended and washed 3 times in distilled water. The pellets were drained, dried and the wet weights determined. A 1:5 ratio (cell weight:distilled water) was prepared for the next step in which the cell suspension was put through a Carver Laboratory press at 15-20 K lbs/square inch and then spun at 5 K for 5 minutes.

(5) The supernatant obtained was saved and sonicated with a microprobe for 8-15 second bursts (Sonifier Cell Disruptor, Heat Systems Co.).

(6) The supernate was then filtered through Whatman #1, Millipore 8 um 0.8 um and 0.45 um filters. The final 0.45 m filtration was to sterilize the extract and the resulting filtrate was aseptically transferred to small sterile tubes in 1 ml aliquots and frozen at -5°C.

(7) The optical density of an aliquot was read at 260 and 280 nm and compared with that of a 10^{-3} concentration of nutrient broth. This was the basis for determining concentration levels to be used in future interaction studies. Prior to addition to samples, aliquots of the extact were re-filtered through a sterile Swinney filter unit fitted with a 0.45 um filter.

B. Late (stationary phase) non-coliform extract:

The above extract is designated "early," the cells still being in logarithmic growth. A second extract termed "late" was prepared as described above, except in step 2, the cultures were grown for 60 hours before harvesting.

C. E. coli extract:

An E. coli extract was also prepared using a frozen paste of E. coli B mid-log phase cells obtained from Grain Processing Co., Muscatine, Iowa. It was prepared as described above.

The bacterial extracts prepared as described above were capable of

supporting the growth of the coliform and non-coliform isolates. The concentration of extract necessary to support the growth of the control organisms was determined for each preparation.

Chemical analysis of bacterial extract--As noted earlier, the amount of bacterial extract added was based on the 260/280 ratio of the extract compared to 10^{-3} nutrient broth. To gain more information on the composition of the extract, the following chemical and biochemical determinations were made.

Total Carbohydrate Determination:

Using a modification of Hassid and Abraham's starch and glycogen analysis(9), it was possible to determine the amount of total carbohydrate (ug/g wet weight) and the amount of glycogen (ug/g) in the bacterial extact.

1. A 2.0 ml aliquot was digested with 1 ml of 60% KOH.
2. 0.1 ml of this solution was brought up to 5 ml with distilled water.
3. One ml and 0.5 ml aliquots were then tested by adding 10 ml Anthrone reagent.
4. Tubes were swirled gently and boiled for 10 min, then immersed in ice and cooled to below room temperature.
5. Readings were done at 620 nm and compared to a standard curve for glucose.

We found 34.4 ug total carbohydrate/g wet weight of extract and 30.87 ug glycogen/g.

Protein Determination:

The amount of protein (ug/ml) was determined using the Bio-Rad method (10).

1. A stock of BSA (60 mg/ml) was appropriately diluted for the standard curve.
2. An aliquot of extract was diluted to fall within a straight line portion of the standard curve.
3. Five ml of Bio-Rad reagent was added to each 0.1 ml of test and standard sample.
4. Samples were read at 595 nm.

One extract prepared 1-4-79 had 41.4 ug protein/ml while another prepared 4-20-79 contained 347.5 ug protein/ml. The 4-79 extract was 6 times more concentrated on a g wet weight/ml extract basis.

DNA Determination:

This method allows the direct measurement of DNA (ug/ml) by fluorescent detection (11). It only works on double stranded DNA. A drawback to this method is that it should be used on freshly broken cells

so there is a minimum of nicking of the DNA by nucleases. Since nuclease inhibitors were not added to the bacterial extracts and since the length of time from the preparation of the extract to the DNA determination varied from days to months, the value of this test is mainly to show the presence of DNA in the extract.

1. The sample should have a 280 nm O.D. of 0.05. This is in a final volume of 1.8 ml.
2. 100 ul of 300mM $MgCl_2$ is added, the tube is shaken, 100 ul of mithramycin is added and the tube is again shaken.
3. Incubate at room temperature 30 min. to 18 hr. We found the greatest stabilization of readings after 18 hrs.
4. Calf thymus DNA at a concentration of 12 ug/ml was used as the standard. In our extracts we had 560 ug DNA/ml. This is about 10% of that expected in freshly sonicated cells.

Supplementation of the bacterial extract--in some experiments the bacterial extract is supplemented. The rationale for these additions is discussed below.

1. Salts + phosphate buffer:

The salt mix + phosphate buffer solution used is the one described in Standard Methods page 888 (2) for testing the purity of distilled water. Since this mix is used when coliform growth is being tested, we thought it might enhance the growth of the coliforms when they were growing in a low nutrient medium.

2. Phosphate buffer:

This serves as a control for the addition of salts + phosphate. If the presence of salts + phosphate did have any effect on the coliform: non-coliform interaction adding back phosphate alone would indicate if the difference was due to the presence of the phosphate buffer.

3. $FeSO_4$:

As is discussed later, it was observed that the salts + phosphate mix did affect the coliform:non-coliform interaction. $FeSO_4$ was added to the bacterial extract to determine if the observed effect was due to the iron containing salt in the mix.

4. Tubercle solids:

Tubercles are present in most water mains. The material from the tubercles may leach into the distribution water thereby becoming available to organisms in the system. Tubercle solids were added to determine if they would in any way affect the coliform:non-coliform interaction.

The effect of various factors on the coliform:non-coliform interaction

The following factors were considered:

a. the specific non-coliform
b. the ratio of coliforms to non-coliforms
c. the growth medium used
d. the presence or introduction of other factors believed to be potentially stressing such as the isolation media.

The specific experiments which contributed to our information on coliform:non-coliform interactions are listed in Table 3.

TABLE 3. INTERACTION EXPERIMENTS

Number	Organisms	Media or Conditions	Figures
1.	*E. coli* and *Flavobacterium* sp.	Bacterial extract	1 - 6
2.	*E. coli* and *Flavobacterium* sp.	Bacterial extract +salts mix + phosphate buffer	7 - 12
3.	*E. coli* and *Flavobacterium* sp.	Bacterial extract + phosphate buffer	13 - 18
4.	*E. coli* and *Acinetobacter* sp.	Bacterial extract	19
5.	*E. coli* and *Acinetobacter* sp.	Bacterial extact + salts mix + phosphate buffer	20
6.	*E. coli* and *Arthrobacter* sp.	Bacterial extract	21 - 23
7.	*E. coli* and *Arthrobacter* sp.	Bacterial extract + salts mix + phosphate buffer	24 - 26
8.	*E. coli* and *Arthrobacter* sp.	Bacterial extract, Bacterial extract + tubercle solids	27 - 30
9.	*E. coli* and *Flavobacterium* sp.	Bacterial extract, physiologically altered *Flavobacterium* sp.	31 - 32
10.	*E. coli* and *Arthrobacter* sp.	Bacterial extract, grown at 6.6°C.	33 - 34

Standard Conditions for Interaction Experiments

Numerous experiments were done in the course of our study to determine the conditions and procedures for running the interaction experiments. The procedures adopted and used throughout the remainder of our work are detailed below.

1. 250 ml flasks were filled with 100 ml of double distilled deionized water and then autoclaved.

The media used and the ratio of coliforms:non-coliforms initially inoculated were both found to have a profound effect on the interactions between the two groups of organisms tested. The day 8 data has been statistically analyzed by comparing the growth rates of the coliforms when they were grown in 1:0, 1:1, 1:10 and 10:1 ratios with non-coliforms. This was done by running an analysis of variance followed, when necessary, by the Student-Newman-Keuls test (12).

When just the coliform control growth rates are compared it is found that they grow best in the bacterial extract supplemented with salts + phosphate, followed by the bacterial extract alone and least well in the extract supplemented with phosphate.

When the coliforms were inoculated into early non-coliform extract alone (1:0 ratio = control) or in 1:1, 10:1, and 1:10 ratios with non-coliforms the profiles in Figures 1-6 were observed. The statistical analysis which compared the growth rates of the coliforms growing in the presence of different numbers of non-coliforms indicated the following:

1:0 (control)	1:1	10:1	1:10

This means that the growth rate of the coliform control was significantly different (at the .05 level) than the growth rates observed when the coliforms were growing in the presence of non-coliforms. When growing in the presence of non-coliforms, the growth rates of the coliforms in 1:1 and 10:1 coliform:non-coliform ratios were not significantly different from each other. In addition, the growth rate of the coliforms in a 1:10 coliform: non-coliform ratio was significantly different from the growth rate of the coliforms when they were in any of the other ratios tested.

In these experiments it was necessary to use numbers of organisms that are higher than those observed in the distribution system in order to more fully characterize the coliform/non-coliform interaction. For example, observation of figures 1-4 indicate that in non-coliform extract when the coliforms are added in equal numbers and even when they are added at approximately a ten fold advantage in numbers over the non-coliforms they are inhibited. Coliform inhibition is more pronounced when the non-coliforms are initially given an approximate ten fold advantage in numbers (Figures 5 and 6). Under these conditions coliforms could not be recovered from the mix by day 4. By using higher densities of non-coliforms it was possible to follow the course of inhibition and determine the difference in coliform recovery when the non-coliform numbers were increased. This data also points up the importance of the determination of total bacterial numbers in water testing as is done by the Standard Plate Count since increased non-coliform levels appear to be a factor in coliform inhibition.

For the early non-coliform extract + salts + phosphate medium (Figures 7 - 12) our statistical analysis indicated the following:

1:0(control)	1:1	1:10	10:1

2. Bacterial extract was added at a concentration previously determined to support growth of the controls.

3. The coliforms and non-coliforms were streaked on PCA plates and incubated overnight. Colonies were scraped into sterile centrifuge tubes with buffered water. Each organism was washed three times. Absorbance was read at 650 nm to determine the number of cells/ml. In those experiments where different ratios of coliforms to non-coliforms were tested, dilutions were made in an attempt to have the following ratios of coliforms:non-coliforms: 1:1, 1:10, 1:0, 0:1, and in some cases 10:1, in the test flasks. We also attempted in all cases to have a total of 1.1×10^3 organisms inoculated per flask.

4. Ratios were run in duplicate. Aliquots from each flask were plated in triplicate.

5. Plates were incubated and counted after 24 - 48 hours.

6. Controls: the flasks containing each organism growing alone (1:0 and 0:1 ratios) served as controls.

In addition to the establishment of standard procedures we had to run experiments every time a new extract was prepared to determine the proper levels of extract to use. When in the course of our experiments we found that the control organisms were not growing as well as they had in previous experiments we had to determine if the extract had been altered with time and was therefore not capable of supporting growth as effectively or if the growth characteristics of the isolate had changed with time. Both situations were observed. These control experiments are not included in this report.

The Effect of Specific Non-Coliforms on Coliforms--

The non-coliforms tested were: *Flavobacterium* sp.
Arthrobacter sp.
Acinetobacter sp.
CDC group II K-1

Under specific conditions which will be expanded upon below we were able to demonstrate inhibition of the coliforms by all of the non-coliforms except for the CDC group II K-1 organism. Our operational definition of inhibition is low levels of growth or recovery of coliforms when they are growing in combination with non-coliforms as compared to recovery or numbers obtained when the coliforms are growing alone in the same media.

The Effect of the Ratio of Coliforms:Non-Coliforms and the Medium Used on Coliform Recovery--

E. coli and *Flavobacterium* sp. interactions were tested in 1:1, 1:10 and 10:1 ratios in non-coliform extract, and the same extract supplemented with an *E. coli* salts mix plus phosphate buffer, and with phosphate buffer alone. The results are graphed in Figures 1 - 18.

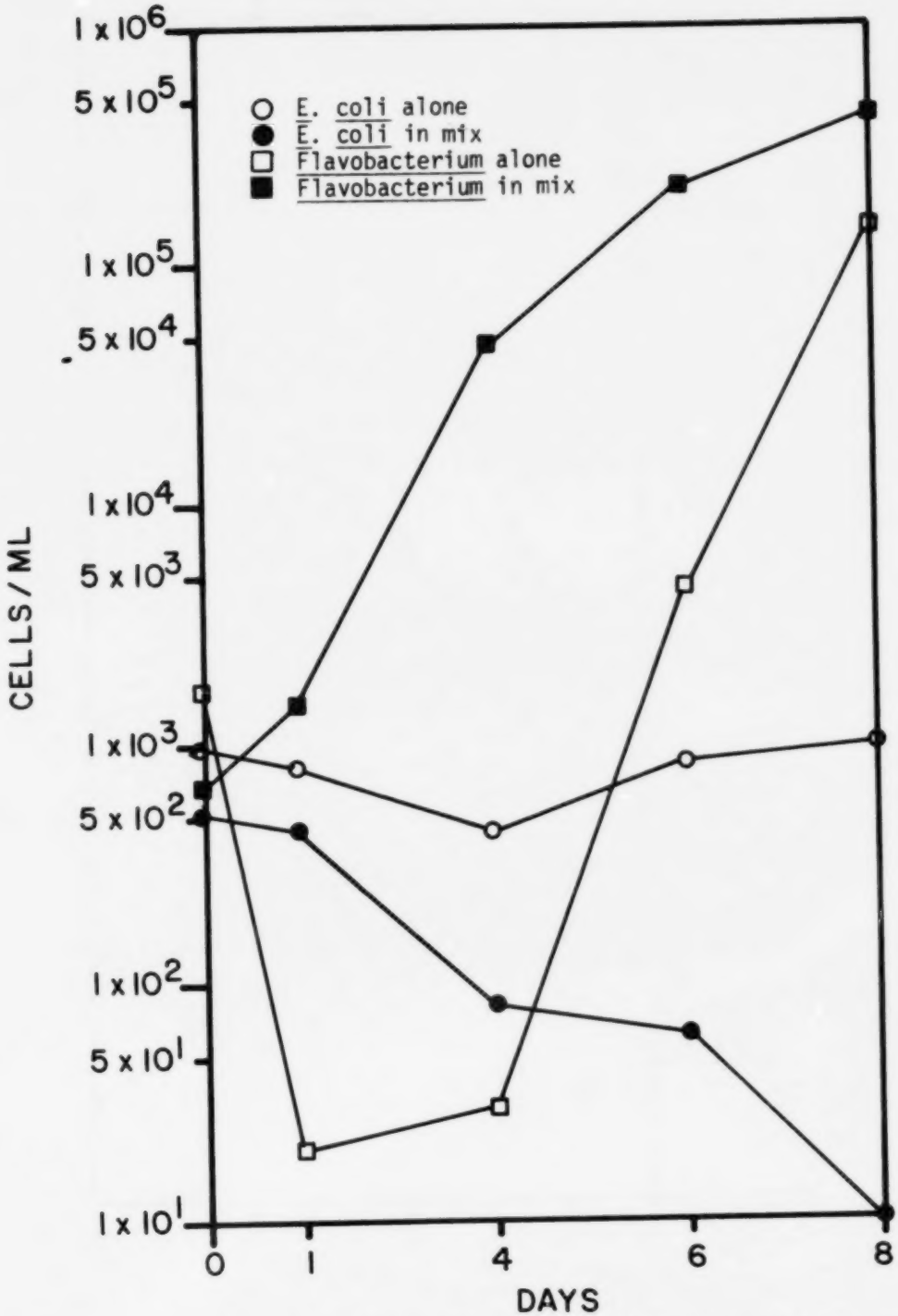

Figure 1. E. coli and Flavobacterium sp. (1:1) in bacterial extract.

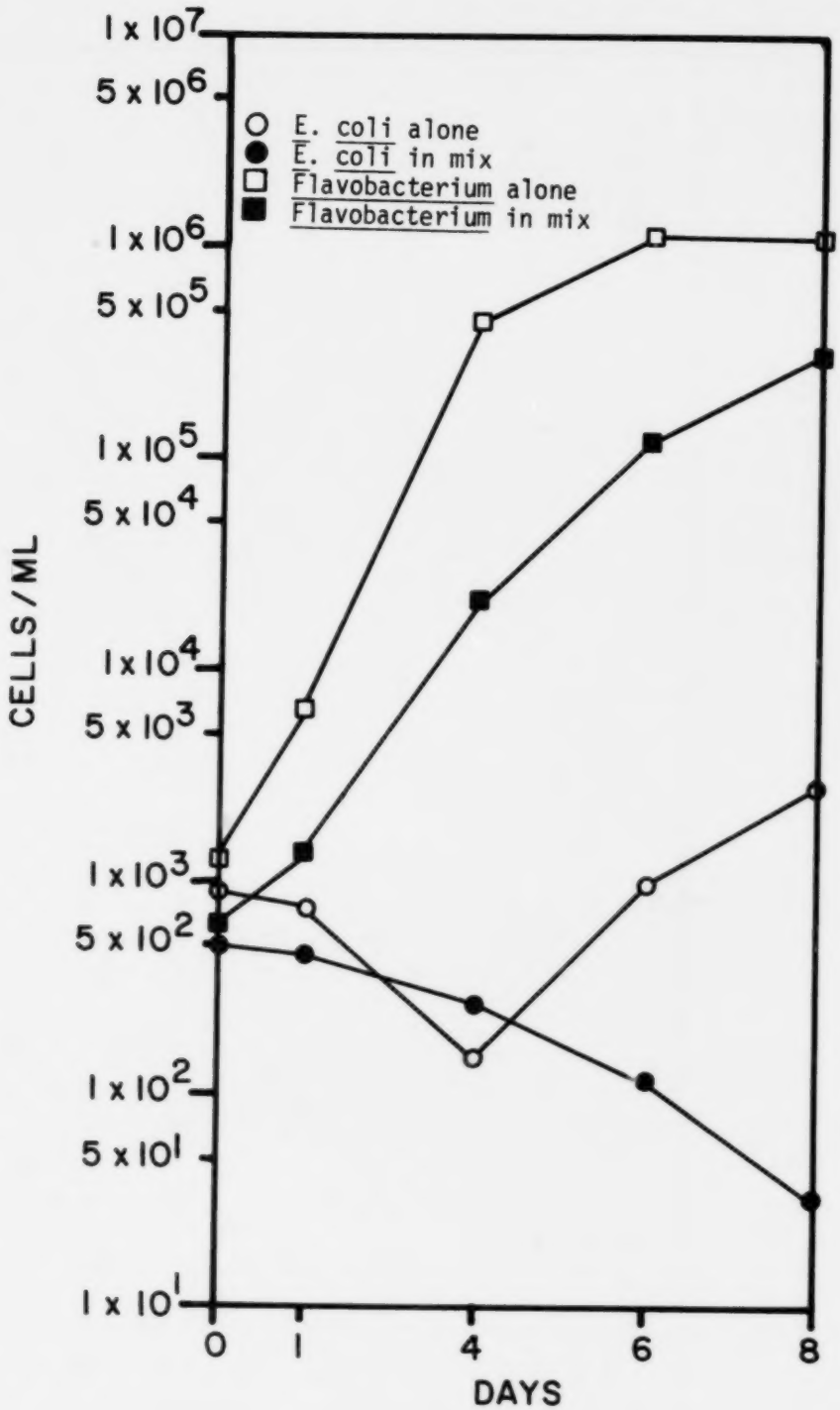

Figure 2. E. coli and Flavobacterium sp. (1:1) in bacterial extract.

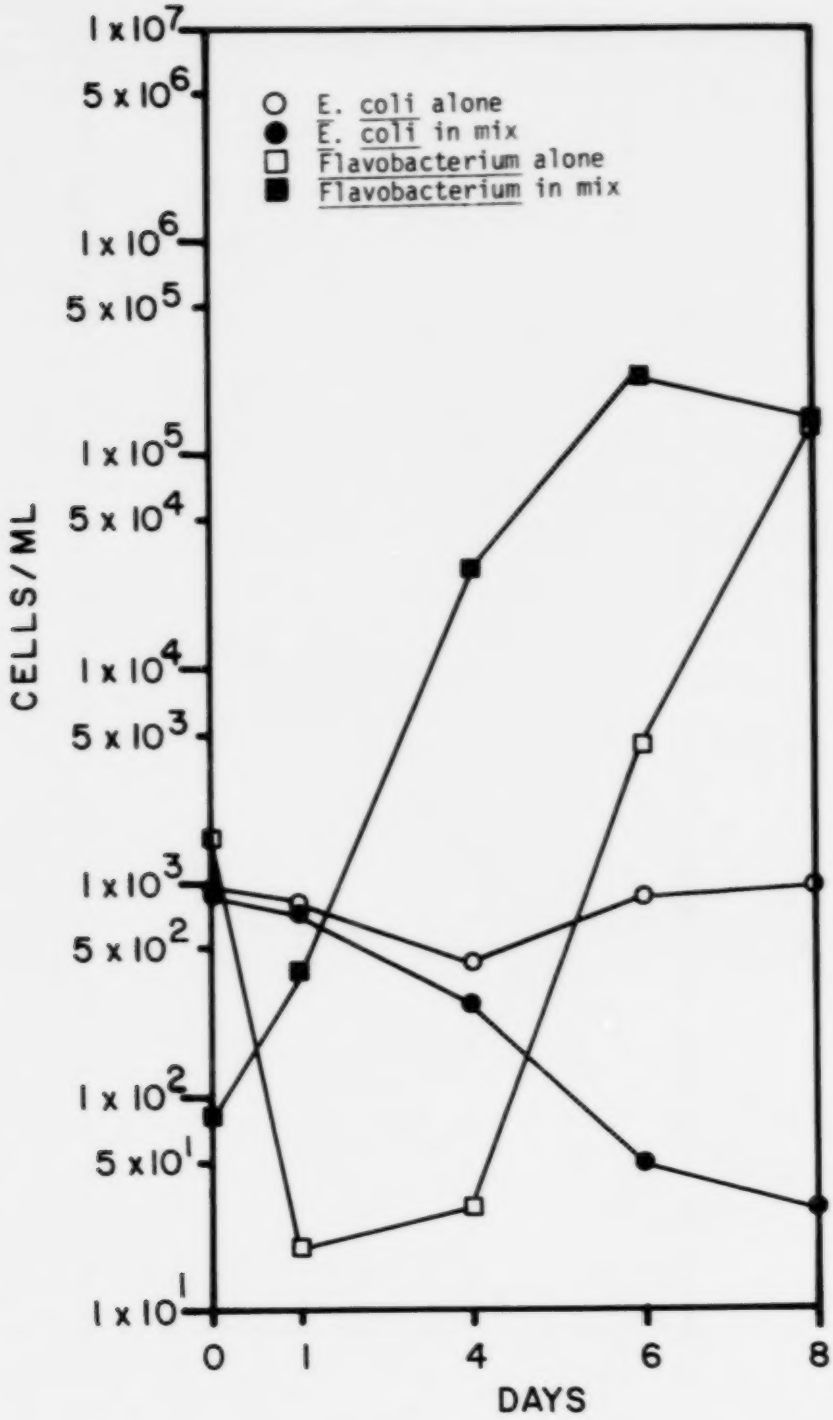

Figure 4. E. coli and Flavobacterium sp. (10:1) in bacterial extract.

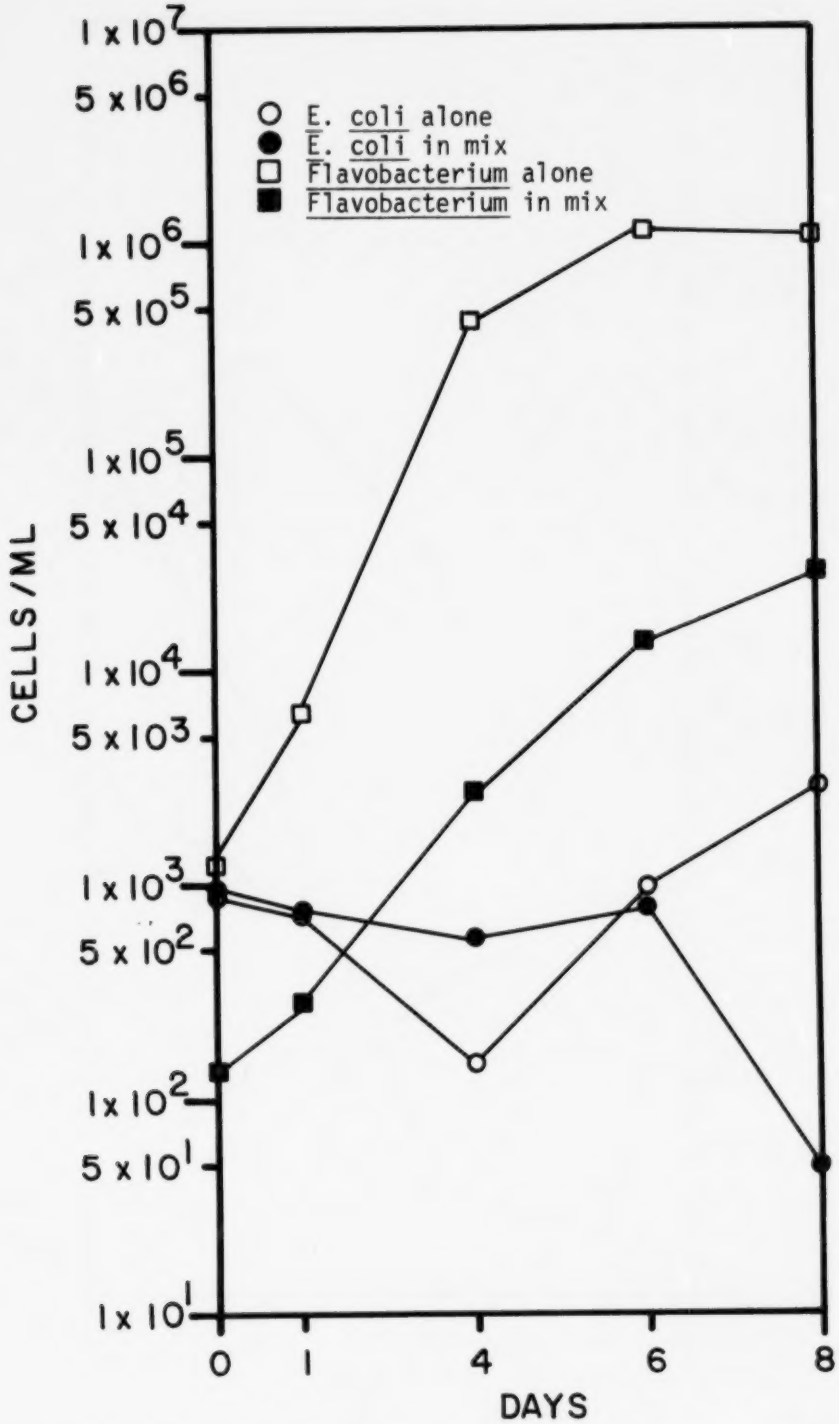

Figure 3. E. coli and Flavobacterium sp. (7:1) in bacterial extract.

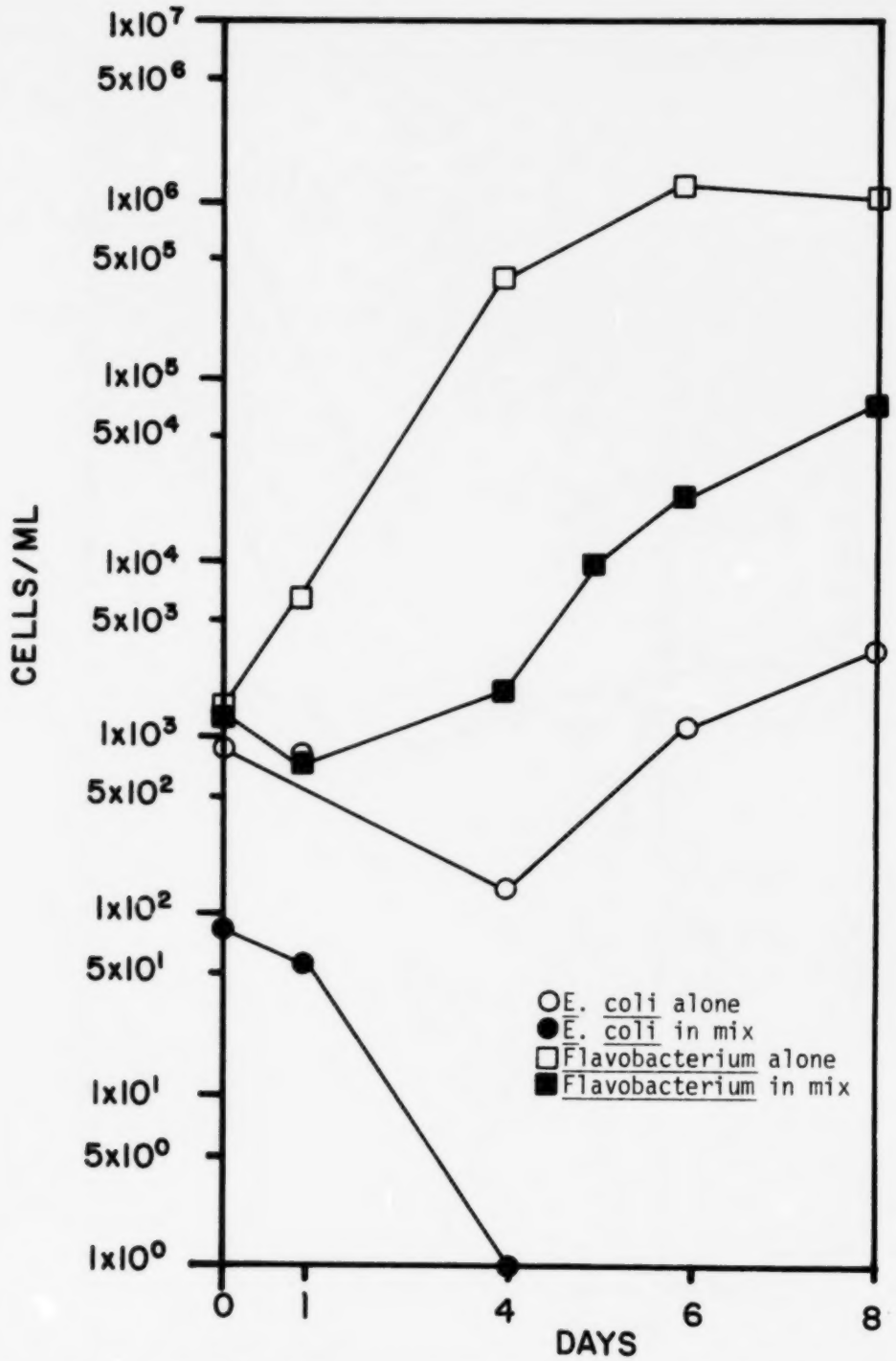

Figure 6. E. coli and Flavobacterium sp. (1:16) in bacterial extract.

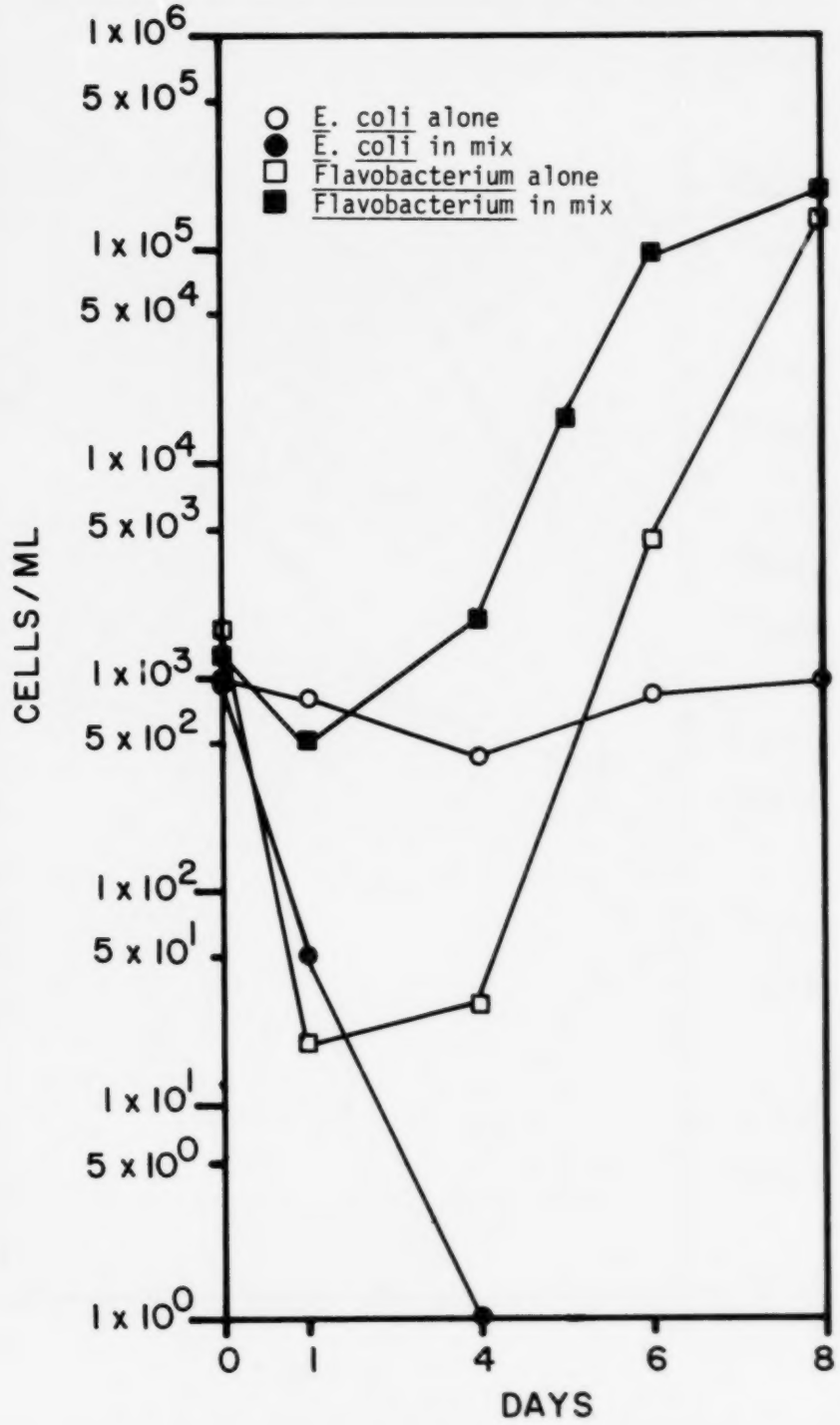

Figure 5. E. coli and Flavobacterium sp. (1:14) in bacterial extract.

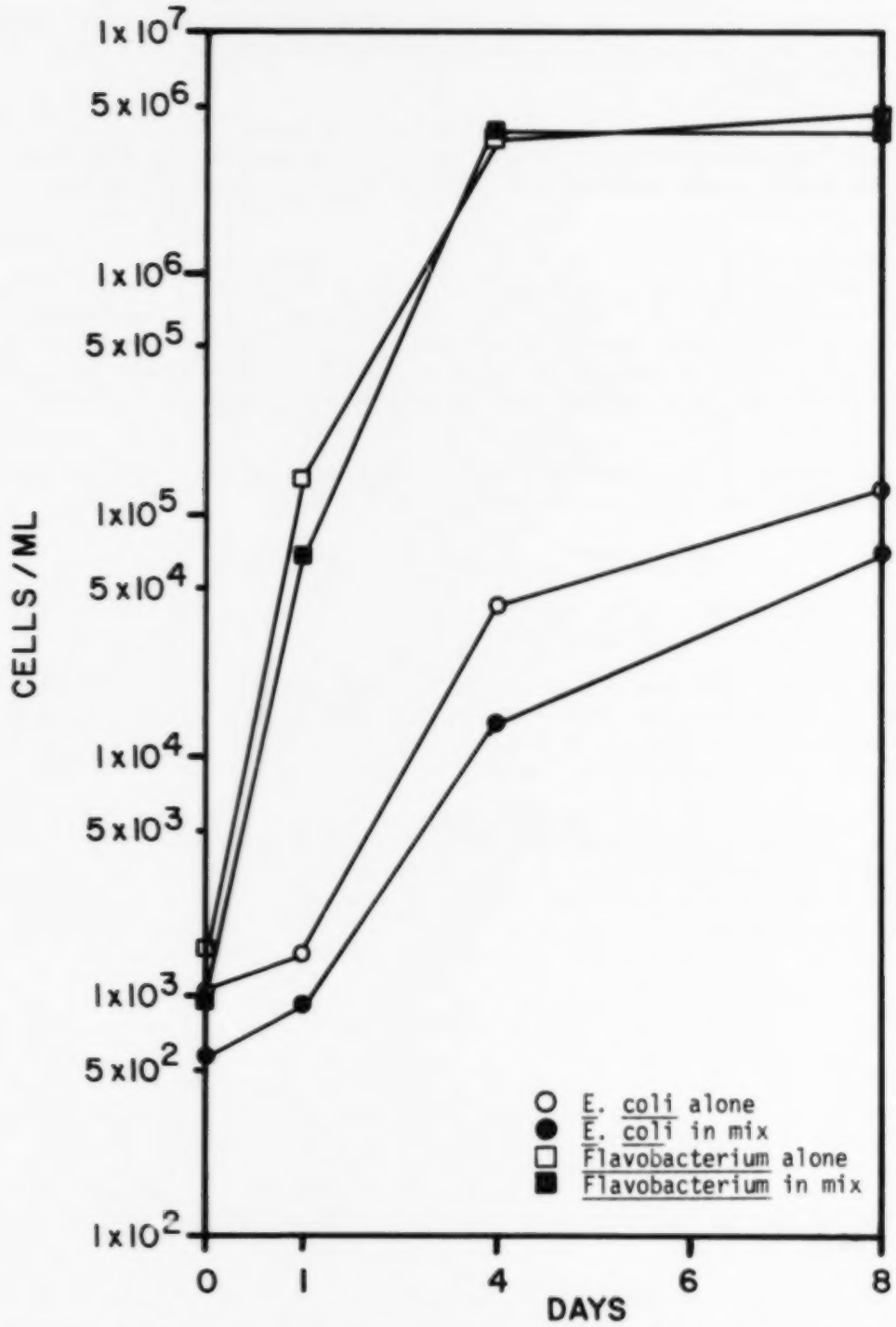

Figure 7. E. coli and Flavobacterium sp. (1:2) in bacterial extract supplemented with salts and phosphate buffer.

This means there was no significant difference in the coliform growth rates when they were grown alone or in combination with non-coliforms in a 1:1 or 1:10 ratio.

When the coliforms were grown in a 10:1 ratio of coliforms:non-coliforms their growth rates were significantly different from the growth rates observed for the coliforms in the other ratios tested.

Observation of figures 7, 8, 9 and 10 indicates that when non-coliform extract is supplemented with salts + phosphate the coliforms are not inhibited by the non-coliforms when the two are grown together in a 1:1 or 1:10 coliform: non-coliform ratio. When the coliforms are given an initial ten fold advantage in numbers under these conditions they are not inhibited and their growth rate in the mix exceeds that of the control (Figures 11 and 12).

For the early non-coliform extract + phosphate medium (Figures 13 - 18) our statistical analysis indicated the following:

1:0 (control)	1:1	1:10	10:1

This means there is no significant difference in the growth rates of the coliforms in the different ratios tested.

This experiment was a control for the addition of salts + phosphate to see if the protective effect on the coliforms of the salts + phosphate supplementation was due to the phosphate buffer. Observation of Figures 13 -18 and comparing them to Figures 7 - 12 indicates this is not the case.

The growth of E. coli and Acinetobacter sp. was tested on bacterial extract and this extract supplemented with salts + phosphate (Figures 19 and 20). The unsupplemented extract supported high levels of coliform growth but was unable to support the Acinetobacter which was undetectable by day four (Figure 19). Growth of each organism in the mix (in a 1:10 ratio of coliforms: non-coliforms) was comparable to that of its respective control. When the medium was supplemented with salts + phosphate (Figure 20) the Acinetobacter control growth equalled the E. coli control growth. In a 1:10 coliform: non-coliform mix the Acinetobacter growth equalled control levels but the coliforms were inhibited. An interesting aspect of this interaction is that the inhibition of the coliform under the conditions of this experiment appear to decrease with time. On day four there is a 10^3 difference in the coliform levels reached in the control and test flasks and by day eight there is only a fifteen fold difference in the levels. It appears that this medium may be conferring a protective effect on both members of the interaction.

Another study on interactions between a coliform and a non-coliform using bacterial extract alone and supplemented with salts + phosphate was done with E. coli and an Arthrobacter sp. (Figures 21-26). In this experiment Violet Red Bile Agar (VRBA), a selective medium, was used to

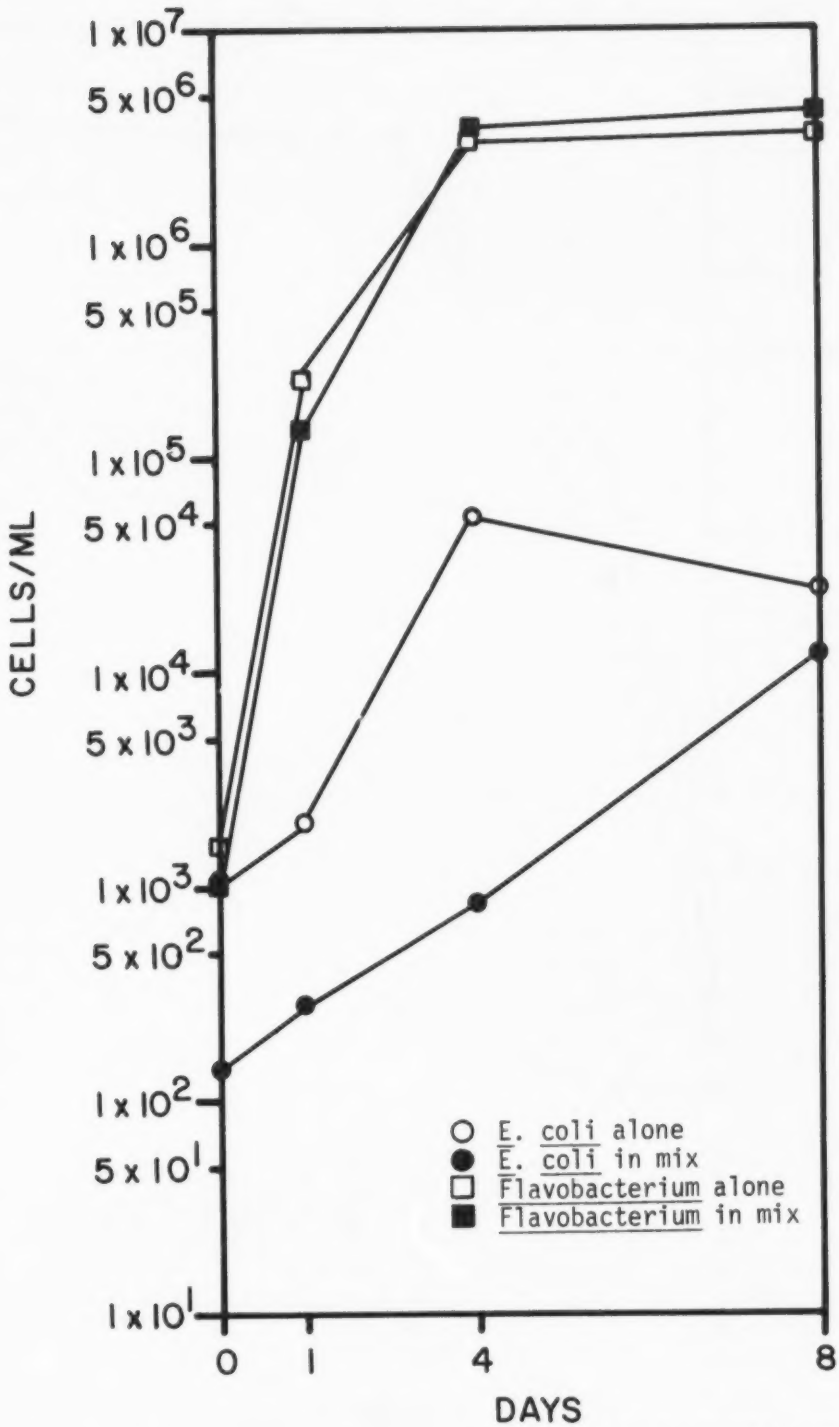

Figure 9. E. coli and Flavobacterium sp. (1:8) in bacterial extract supplemented with salts and phosphate buffer.

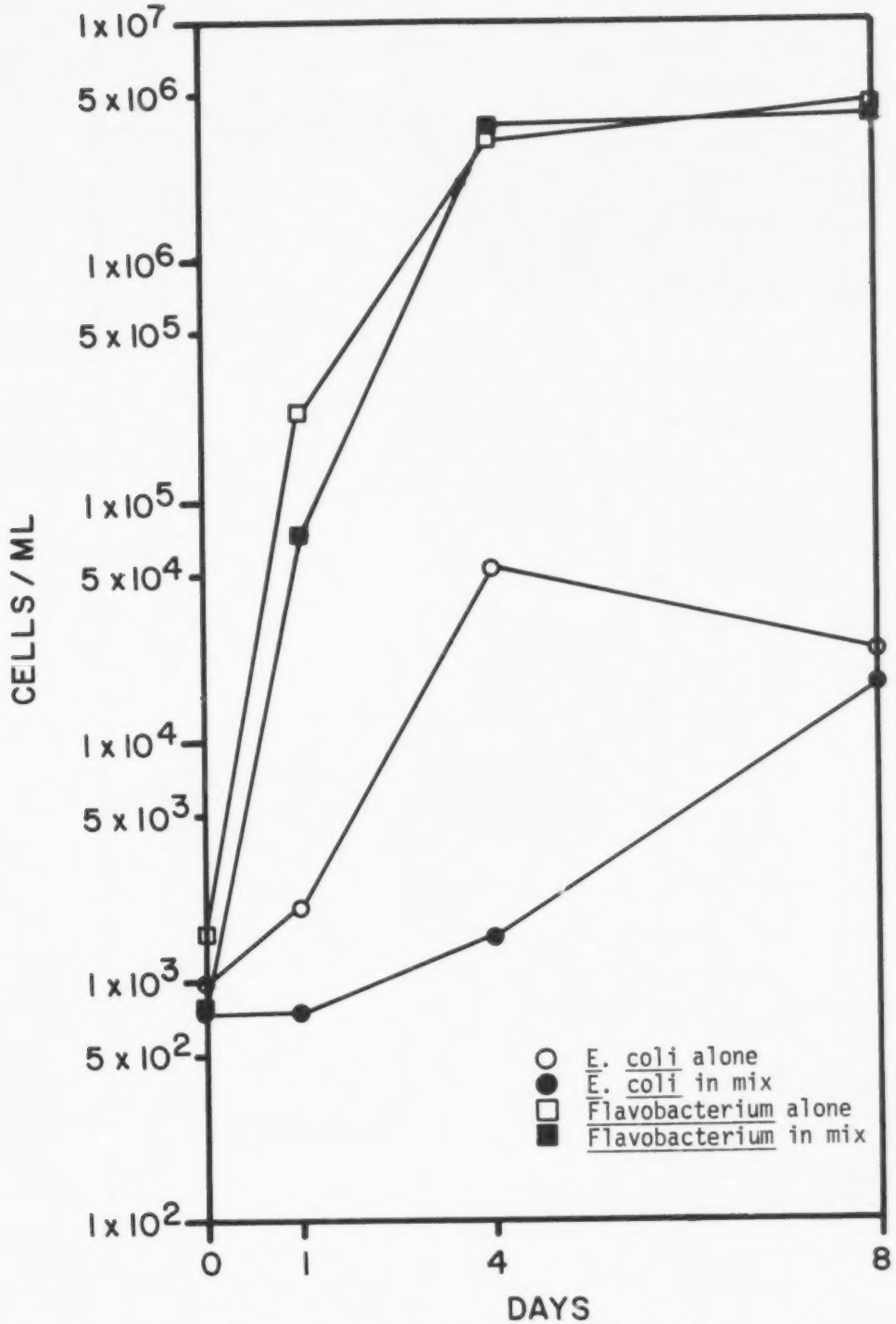

Figure 8. E. coli and Flavobacterium sp. (1:1) in bacterial extract supplemented with salts and phosphate buffer.

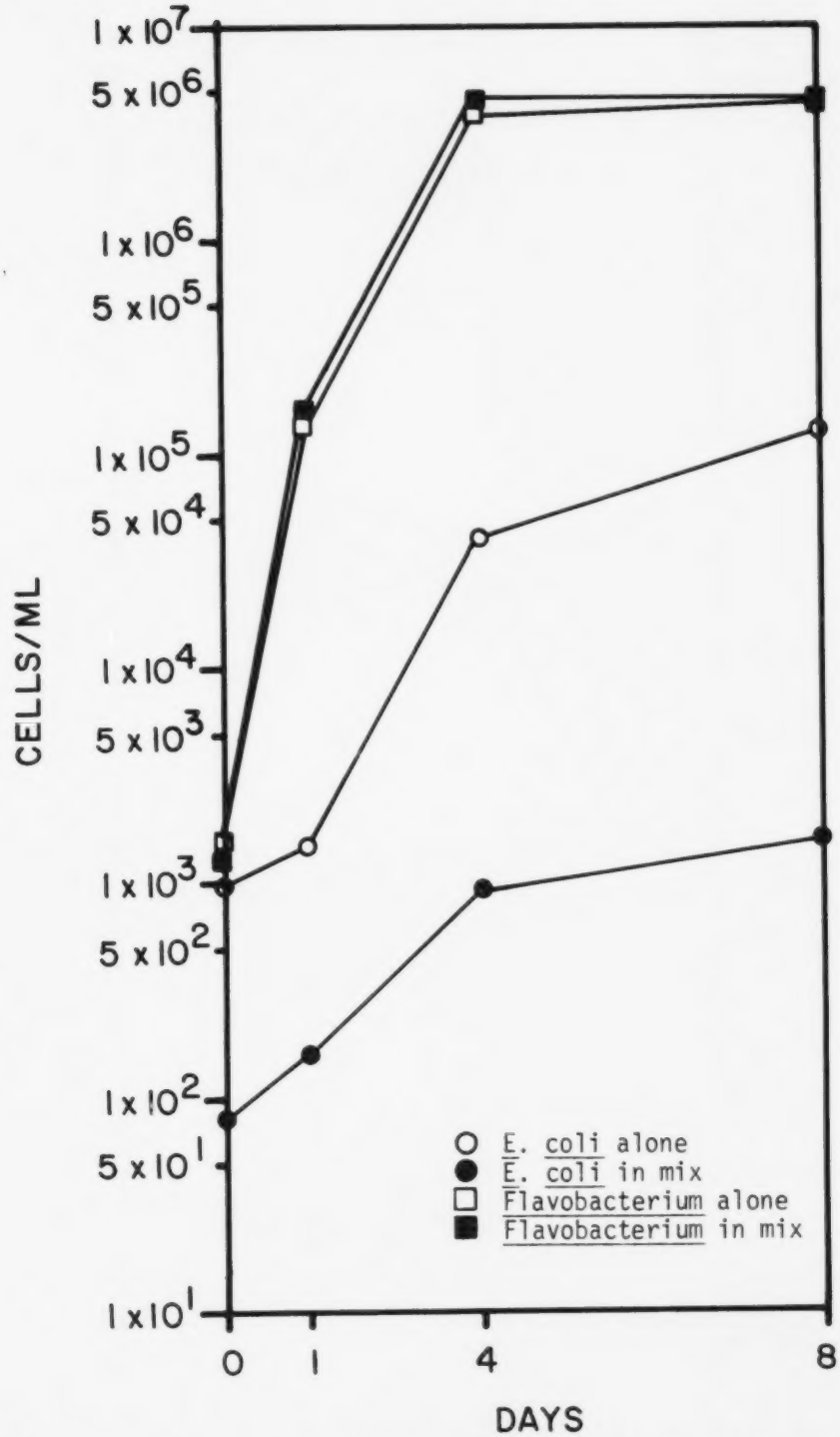

Figure 10. E. coli and Flavobacterium sp. (1:16) in bacterial extract supplemented with salts and phosphate buffer.

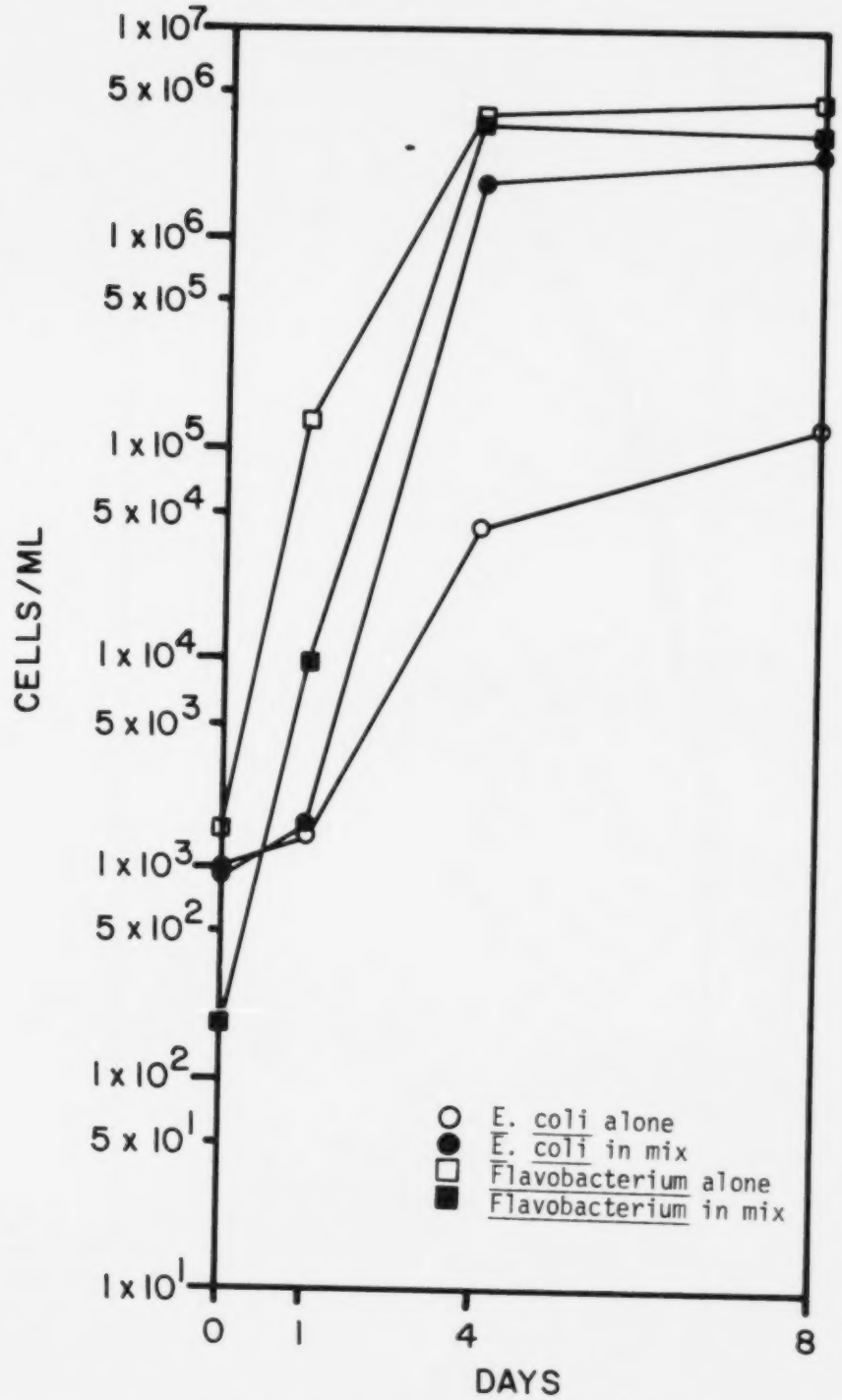

Figure 11. E. coli and Flavobacterium sp. (6:1) in bacterial extract supplemented with salts and phosphate buffer.

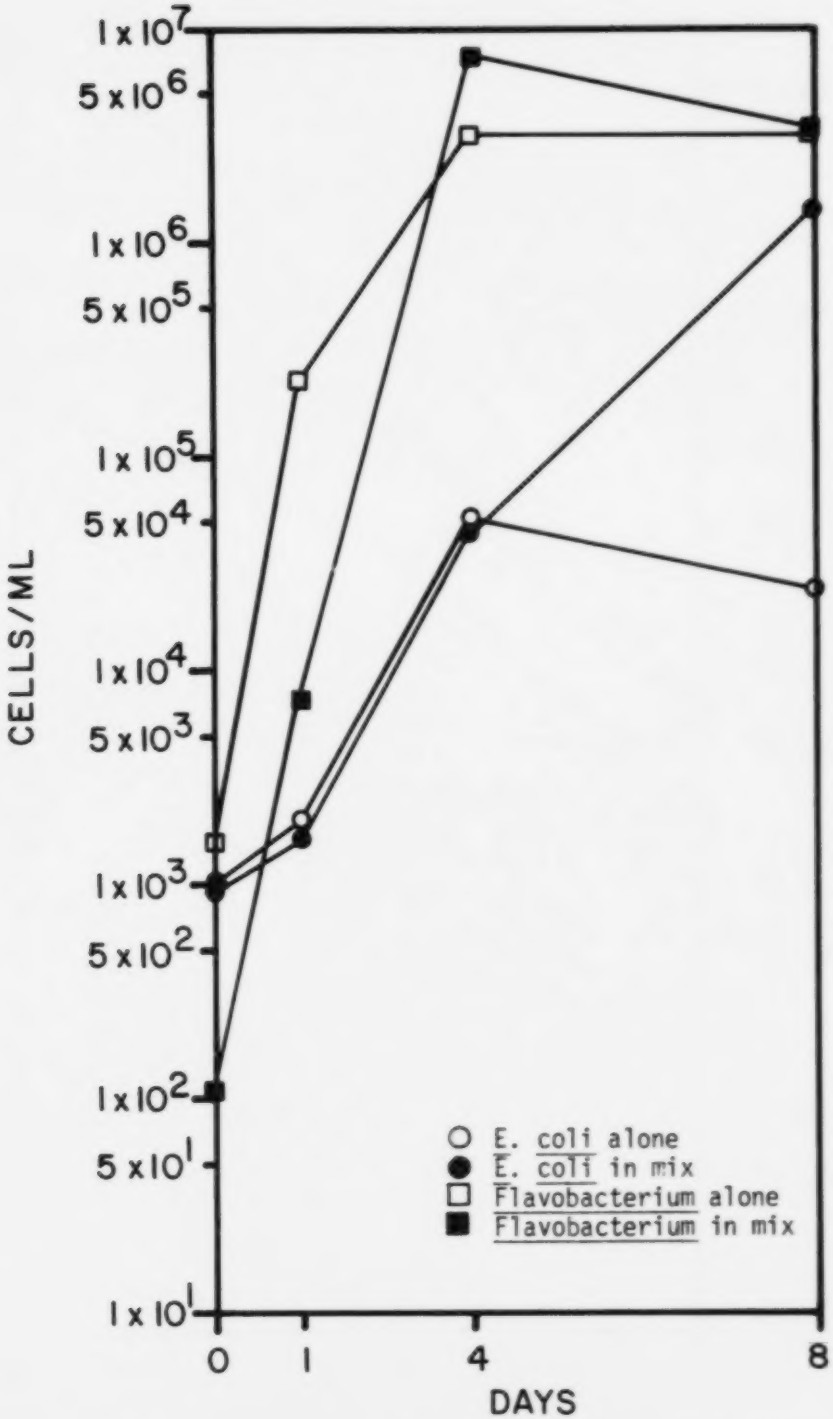

Figure 12. E. coli and Flavobacterium sp. (9:1) in bacterial extract supplemented with salts and phosphate buffer.

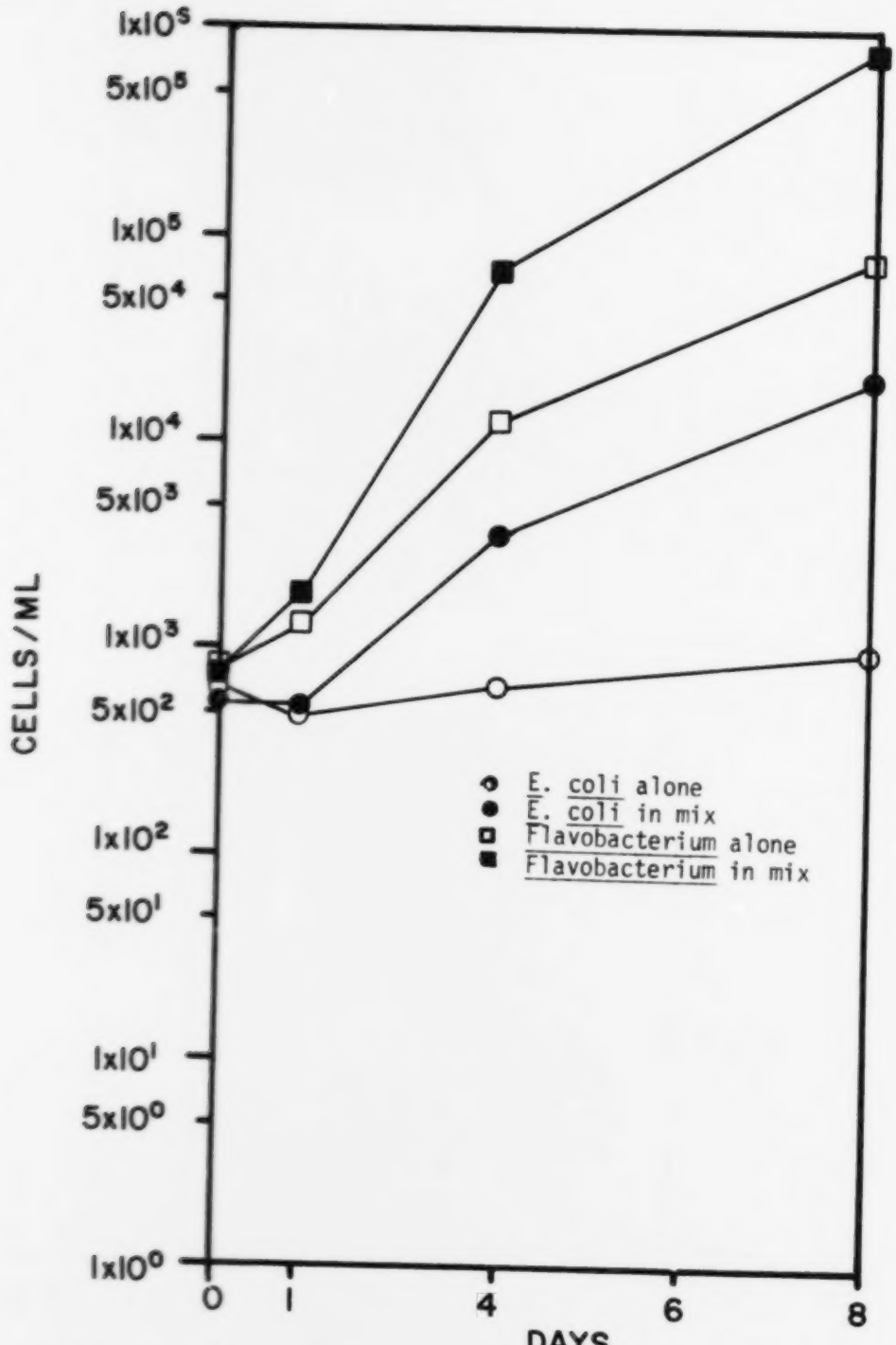

Figure 13. E. coli and Flavobacterium sp. (1:1) in bacterial extract supplemented with phosphate buffer.

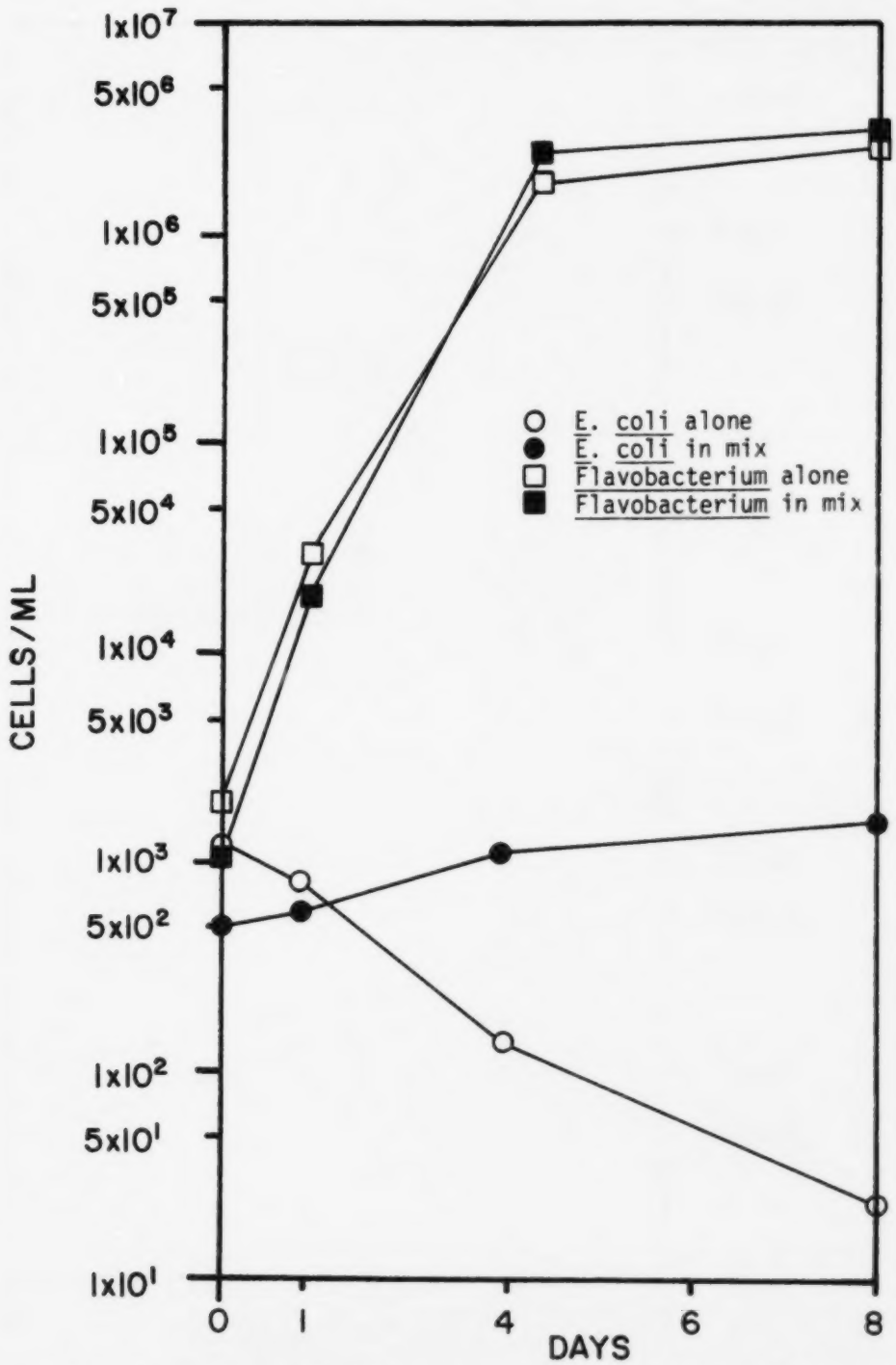

Figure 14. E. coli and Flavobacterium sp. (1:2) in bacterial extract supplemented with phosphate buffer.

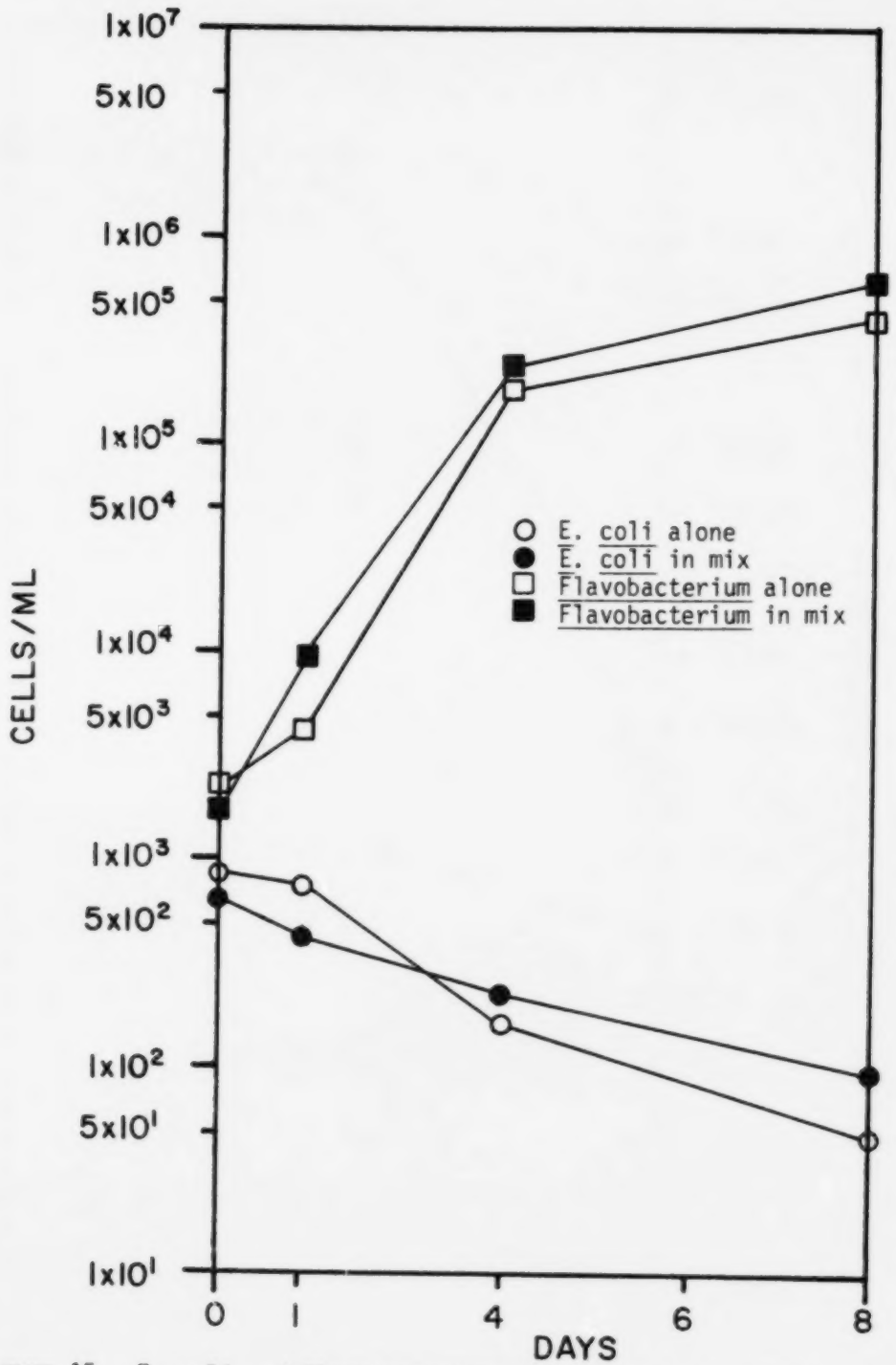

Figure 15. E. coli and Flavobacterium sp. (1:14) in bacterial extract supplemented with phosphate buffer.

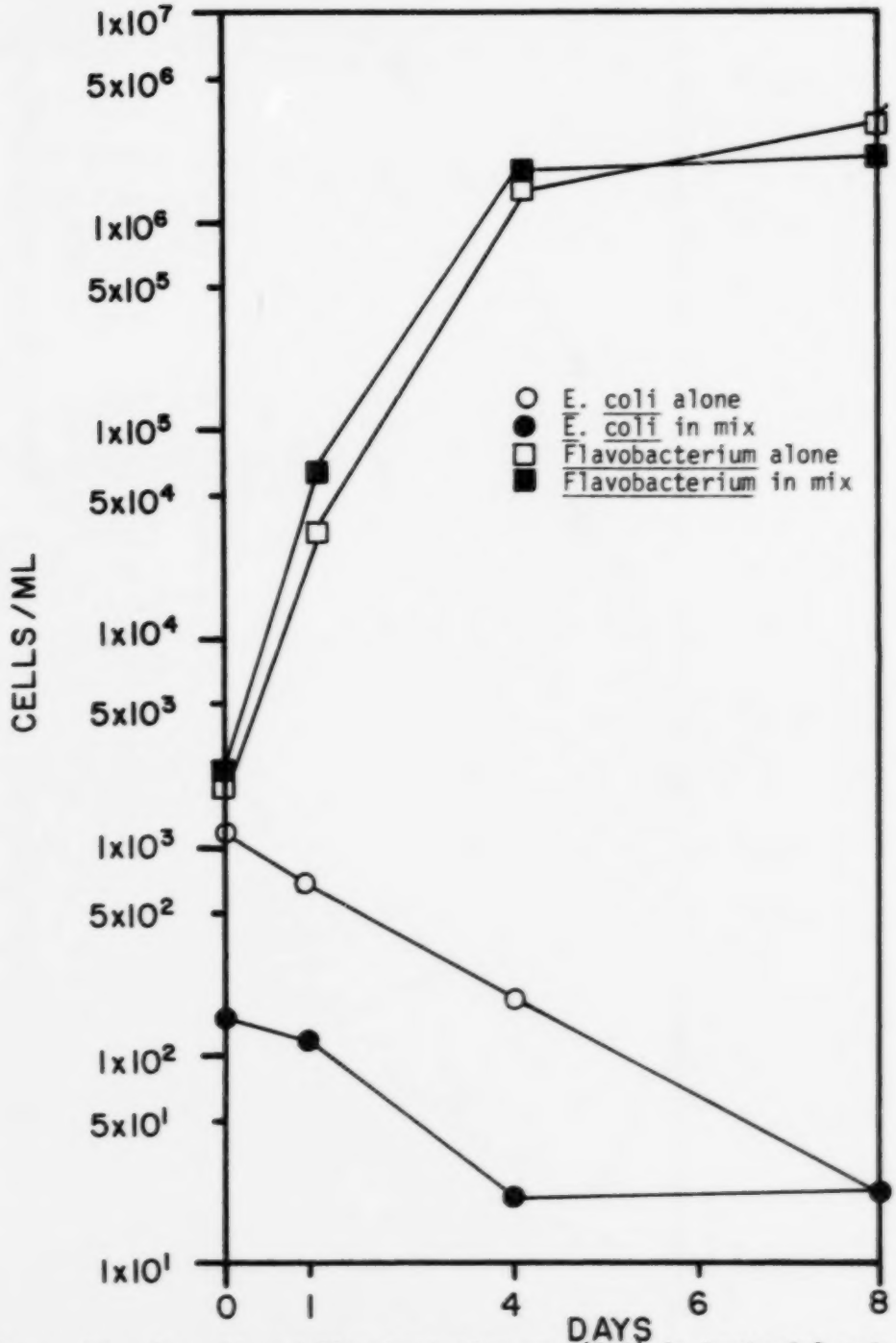

Figure 17. E. coli and Flavobacterium sp. (8:1) in bacterial extract supplemented with phosphate buffer

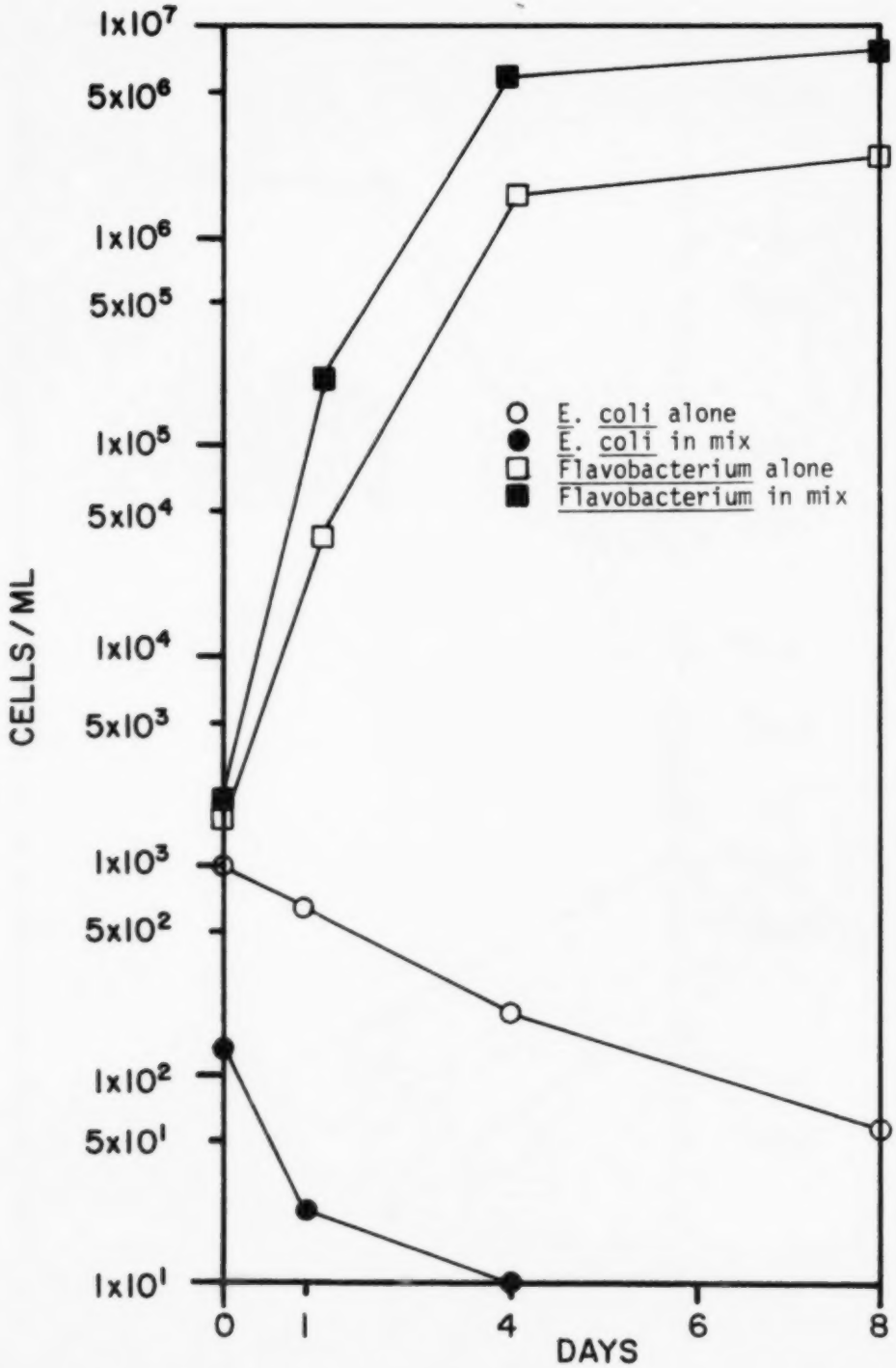

Figure 16. E. coli and Flavobacterium sp. (1:17) in bacterial extract supplemented with phosphate buffer.

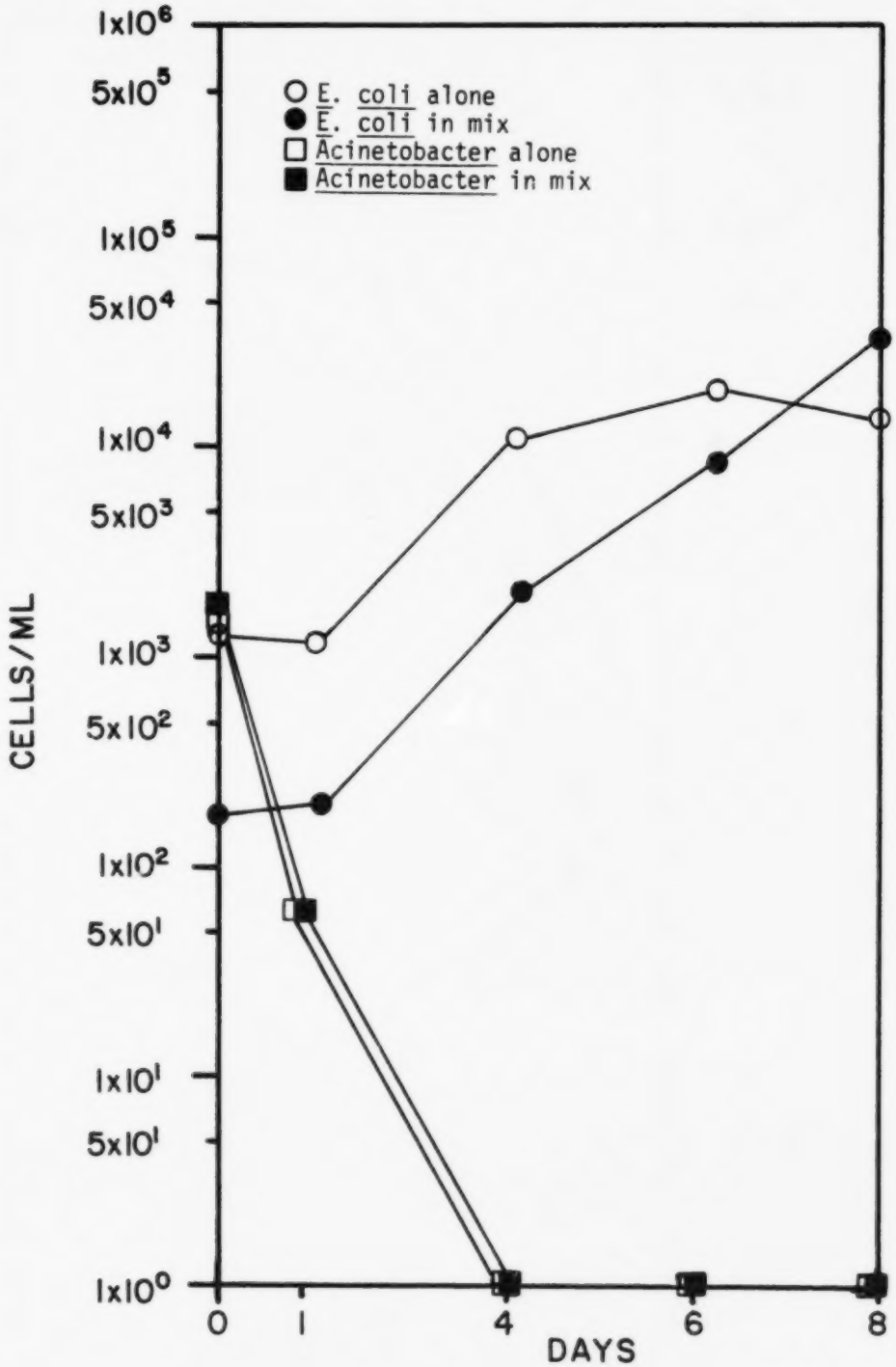

Figure 19. E. coli and Acinetobacter sp. (1:10) in bacterial extract.

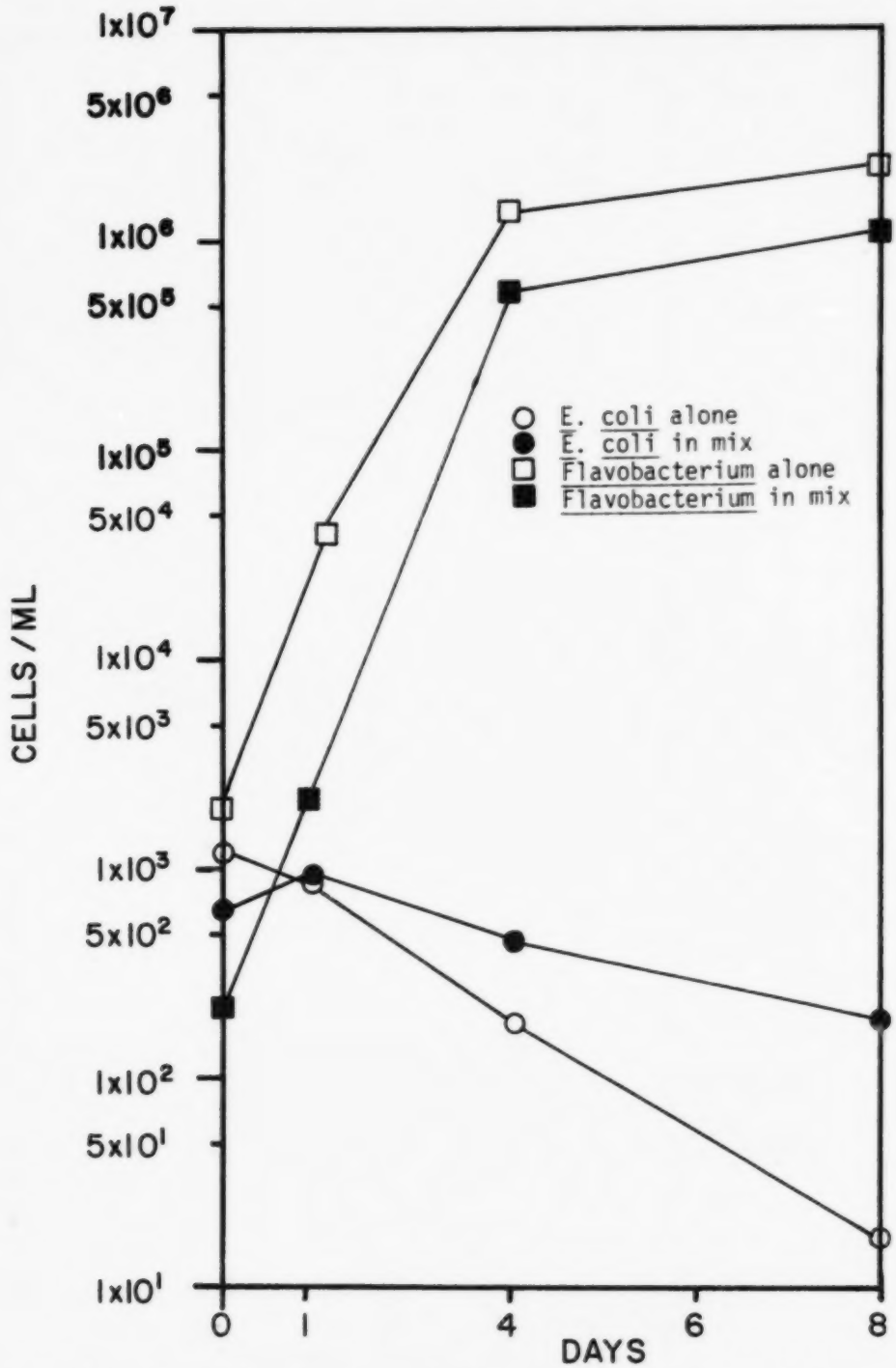

Figure 18. E. coli and Flavobacterium sp. (5:1) in bacterial extract supplemented with phosphate buffer.

enumerate coliforms and to compare coliform recovery with recovery on Plate Count Agar (PCA), a non-selective medium. If the coliforms were stressed, their recovery on VRBA would be less than that on PCA. Using this criteria of stress, the coliform control (Figure 21) was unstressed based on the level of growth attained at 8 days since recovery on VRBA and PCA was similar. When grown in combination in a 1:1 ratio (Figure 22) in non-coliform extract, after an initial lag of four days coliform numbers increase and recovery again is identical on the two media. However, when the ratio of coliform to non-coliform is changed to 1:10 after a similar four day lag when numbers increase on PCA a definite decrease on VRBA is observed (Figure 23). This suggests that the coliforms are stressed.

Addition of salts + phosphate to the non-coliform extract again appears to confer protection on the coliforms (Figures 24-26). Control growth reached higher levels in a shorter period of time on this supplemented medium (Figure 24). As expected recovery on selective and non-selective medium was identical. In a 1:1 ratio with Arthrobacter, coliform numbers approximate control numbers with no indication of stress (Figure 25). In a 1:10 coliform: non-coliform ratio (Figure 26) the protective effect of the salts + phosphate is still evident. Growth levels in the mix again approximate the control and the difference in recovery on the selective and non-selective media is much less pronounced than that observed in the unsupplemented medium (Figure 23).

The above series of experiments demonstrates the protective effect of the salts + phosphate mix on the coliforms in their interaction with non-coliforms. In the distribution system in the presence of unlined mains, iron salts may be introduced from pipe corrosion into distribution water. Tubercles with large numbers of associated bacteria can be isolated from these unlined mains. In the next experiment with E. coli and Arthrobacter we attempted to see if tubercle solids or the iron salt in the salts mix used above ($FeSO_4$) would enhance the growth of E. coli. We also tested the interaction of E. coli and Arthrobacter in the presence of bacterial extract supplemented with tubercle solids.

Neither tubercle solids alone nor $FeSO_4$ alone supported the growth of either isolate (Figures 27 and 28). Finding that the non-coliform extract also did not support the growth of Arthrobacter was surprising and most likely a technical error since a later rerun (Figure 29) of this interaction indicated that the same concentration of extract did support the growth of Arthrobacter. Controls here reached levels of approximately 10^7 organisms/ml. In a 1:10 ratio with coliforms in this extract the coliforms were observed to be inhibited. By day eight there was a 10^3 difference in the levels obtained by the coliforms in the mix when compared to control levels. When the bacterial extract is supplemented with tubercle solids the Arthrobacter control grows well but active growth of the coliform control is not observed. When the two are combined in this medium in a 1:10 coliform: non-coliform ratio (Figure 30), Arthrobacter growth resembles its control growth. Coliform numbers in the mix, as was seen for the control in this medium are barely maintained. The tubercle solids therefore did not appear to enhance the growth or confer protection on the coliforms.

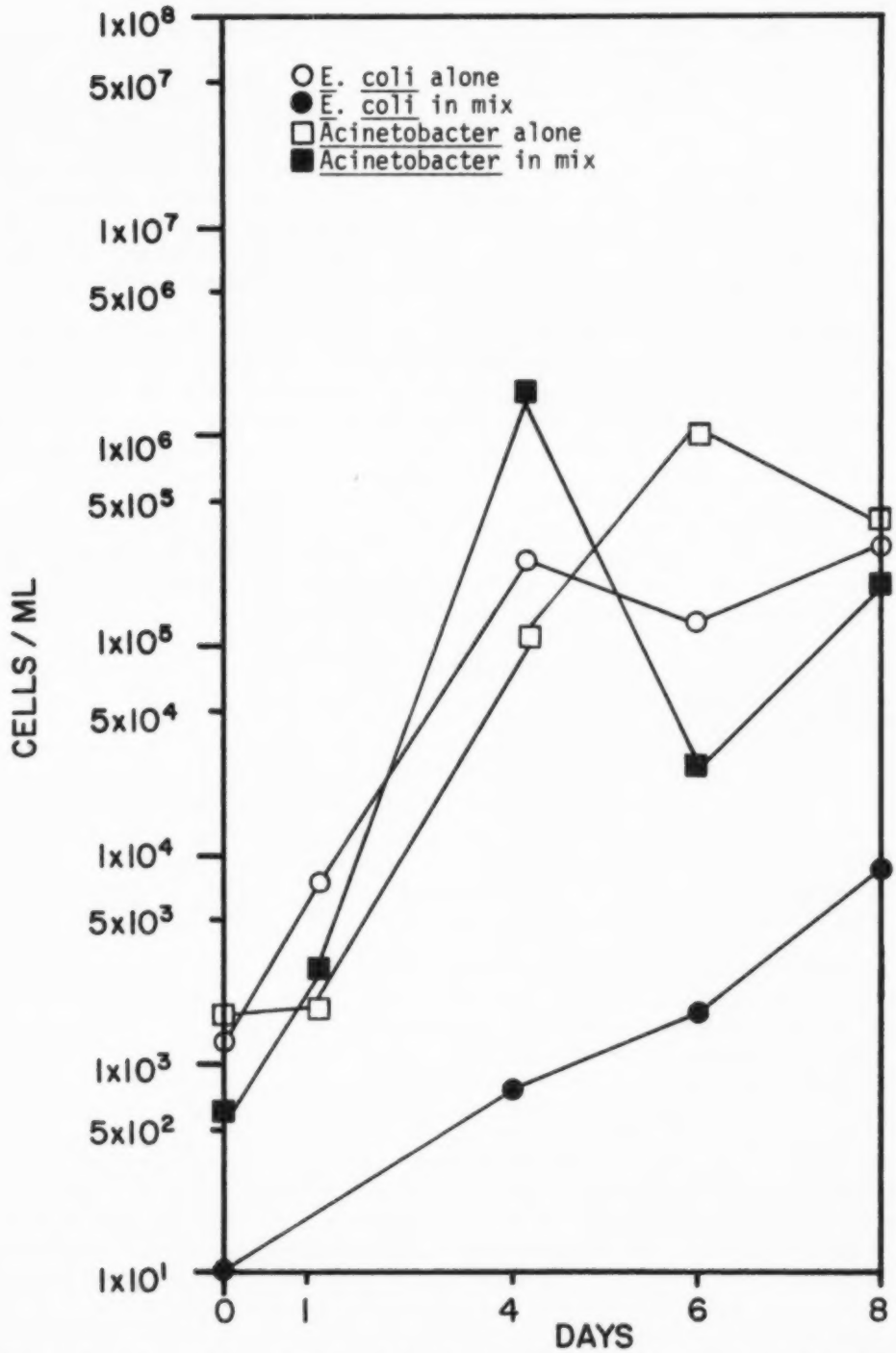

Figure 20. E. coli and Acinetobacter sp. (1:10) in bacterial extract supplemented with salts and phosphate buffer

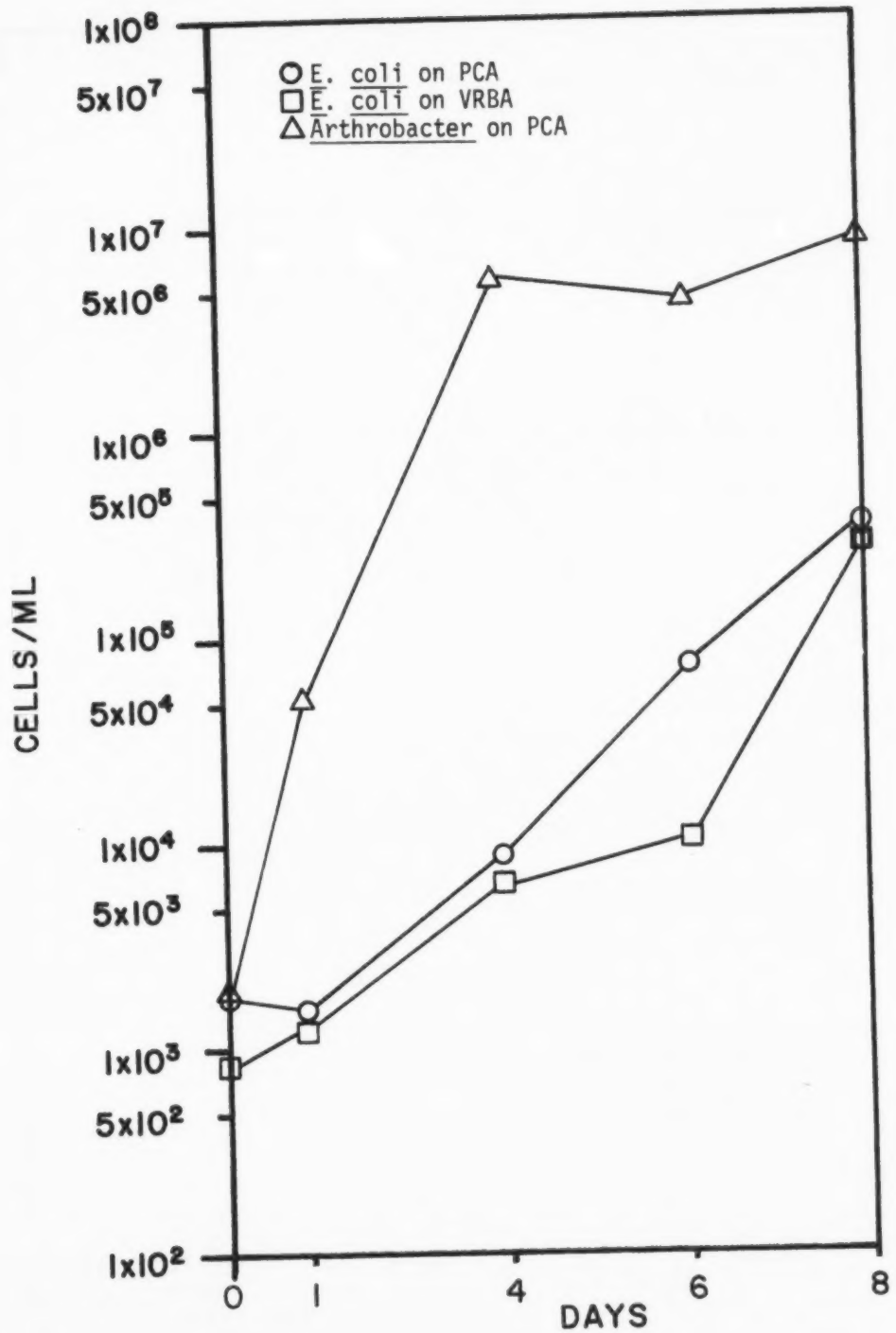

Figure 21. Growth of E. coli and Arthrobacter sp. in bacterial extract.

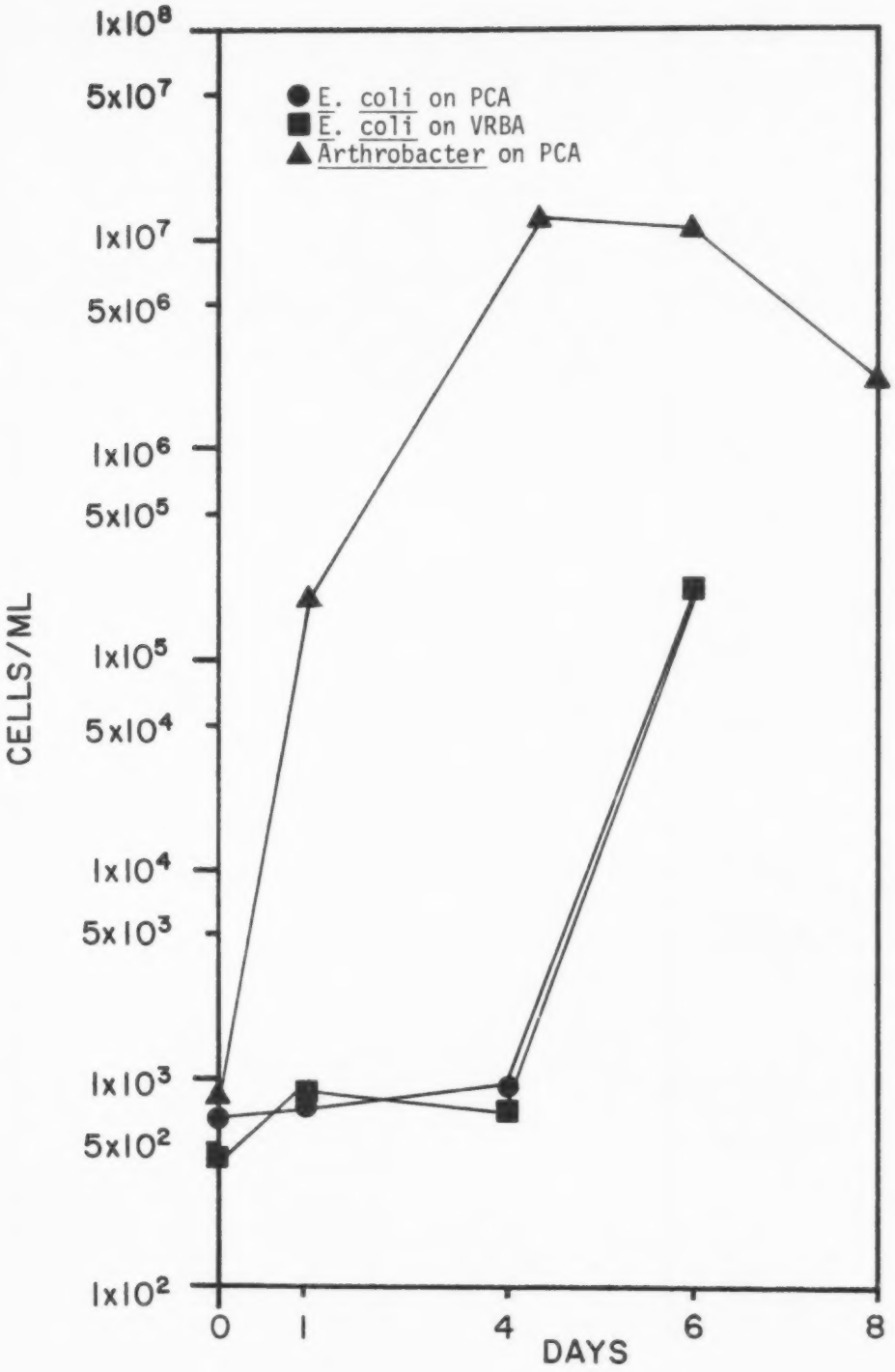

Figure 22. E. coli and Arthrobacter sp. (1:1) in bacterial extract.

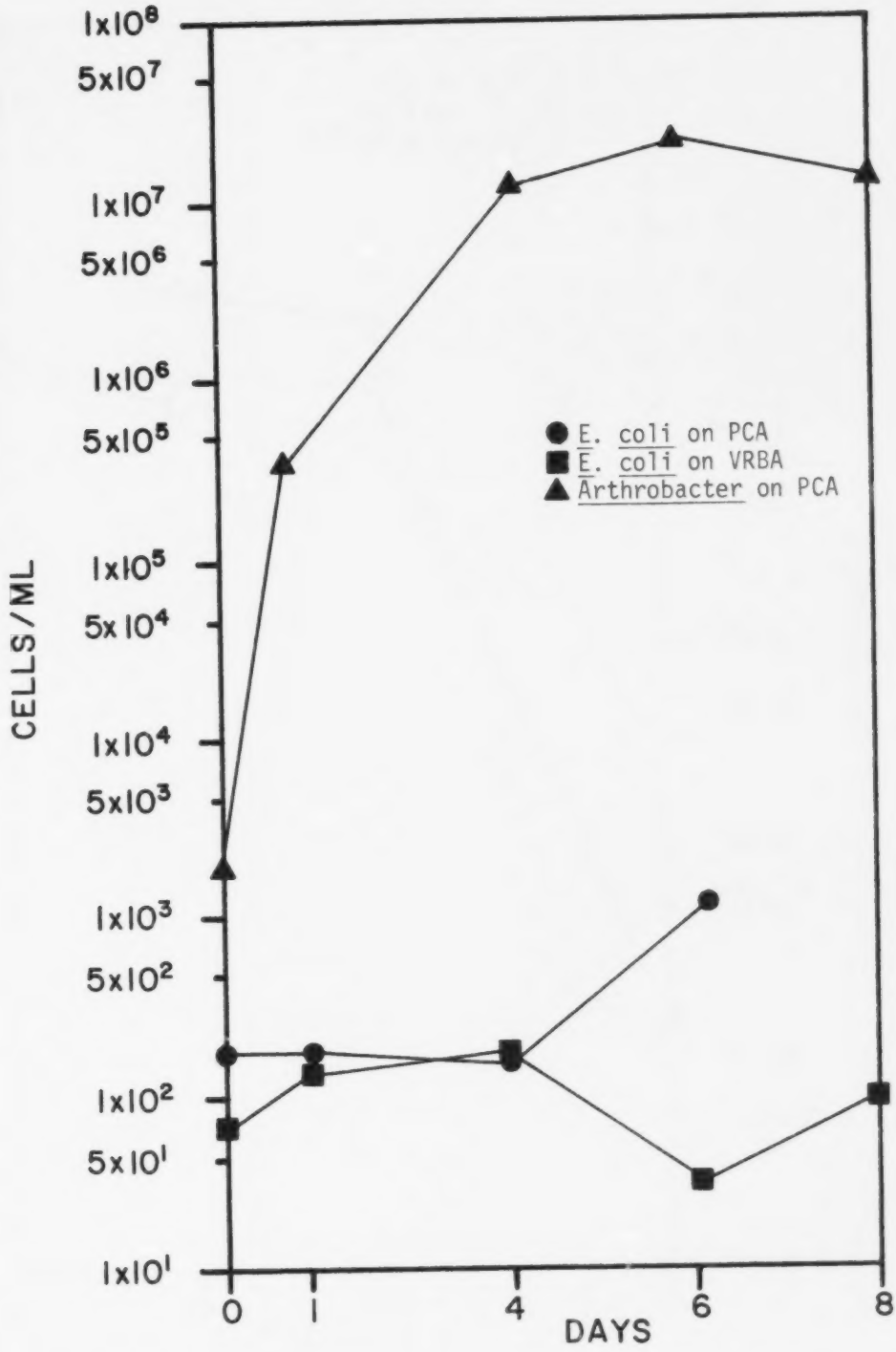

Figure 23. E. coli and Arthrobacter sp. (1:10) in bacterial extract.

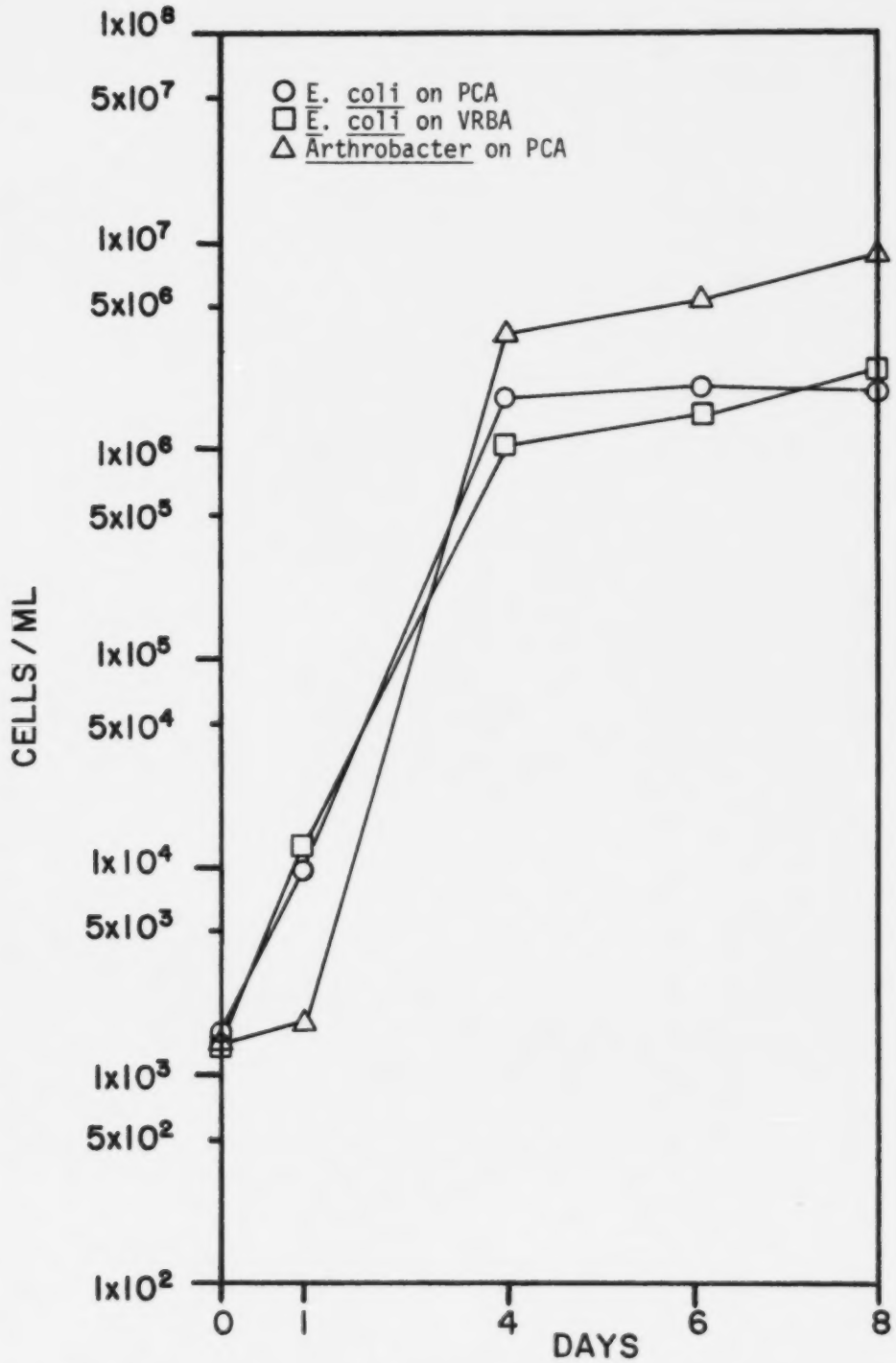

Figure 24. Growth of E. coli and Arthrobacter sp. in bacterial extract supplemented with salts and phosphate buffer.

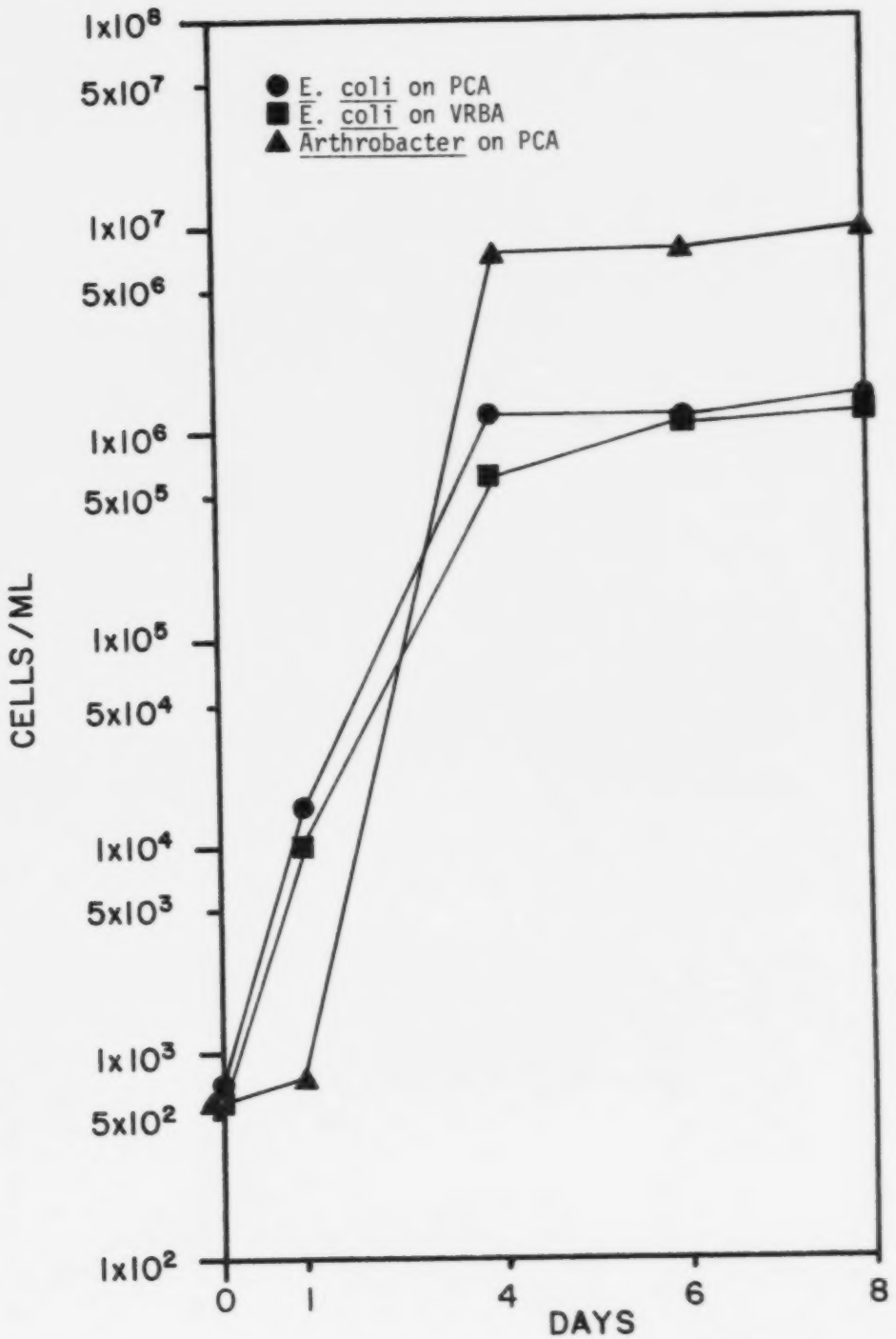

Figure 25. E. coli and Arthrobacter sp. (1:1) in bacterial extract supplemented with salts and phosphate buffer.

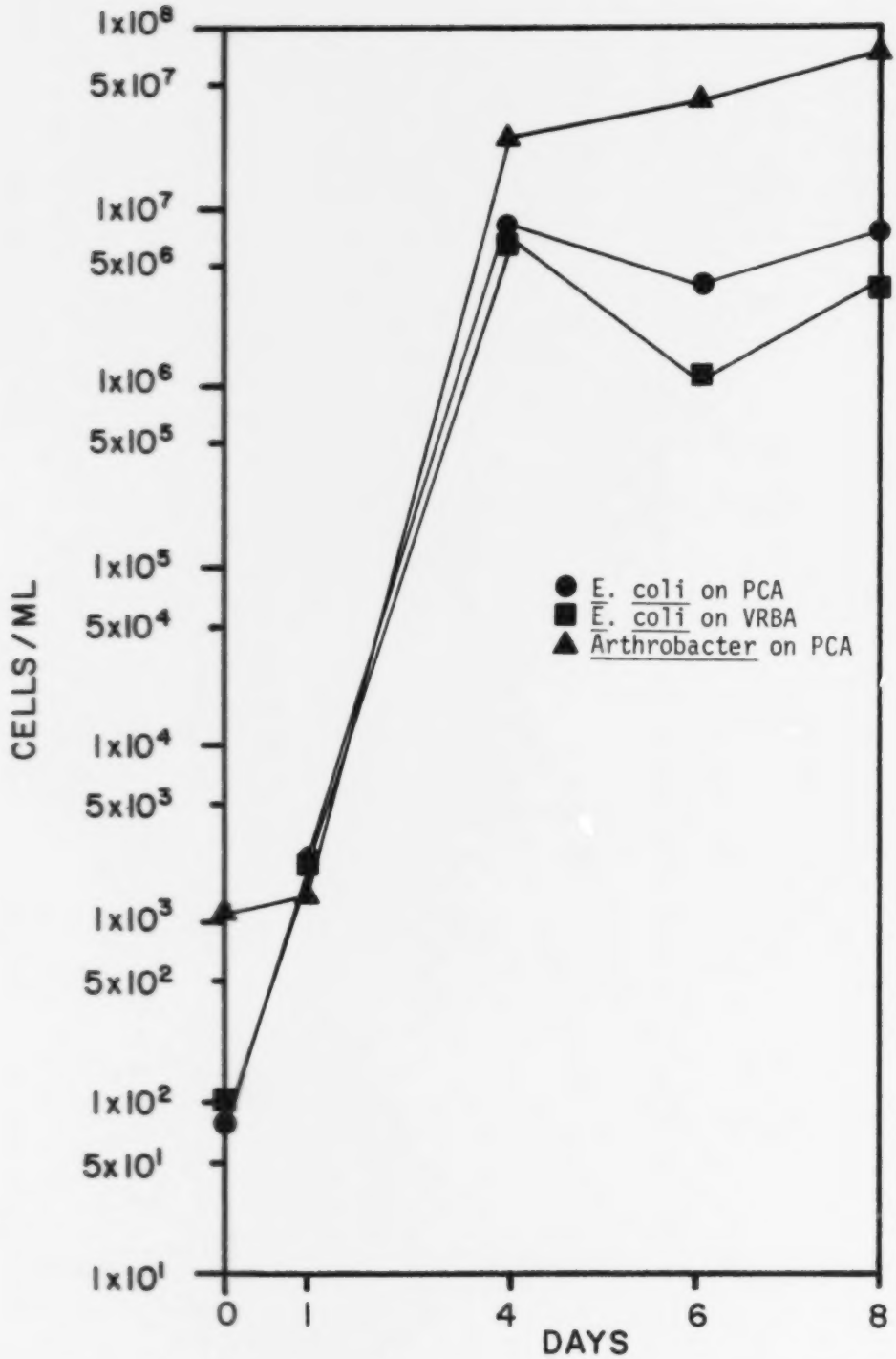

Figure 26. E. coli and Arthrobacter sp. (1:10) in bacterial extract supplemented with salts and phosphate buffer.

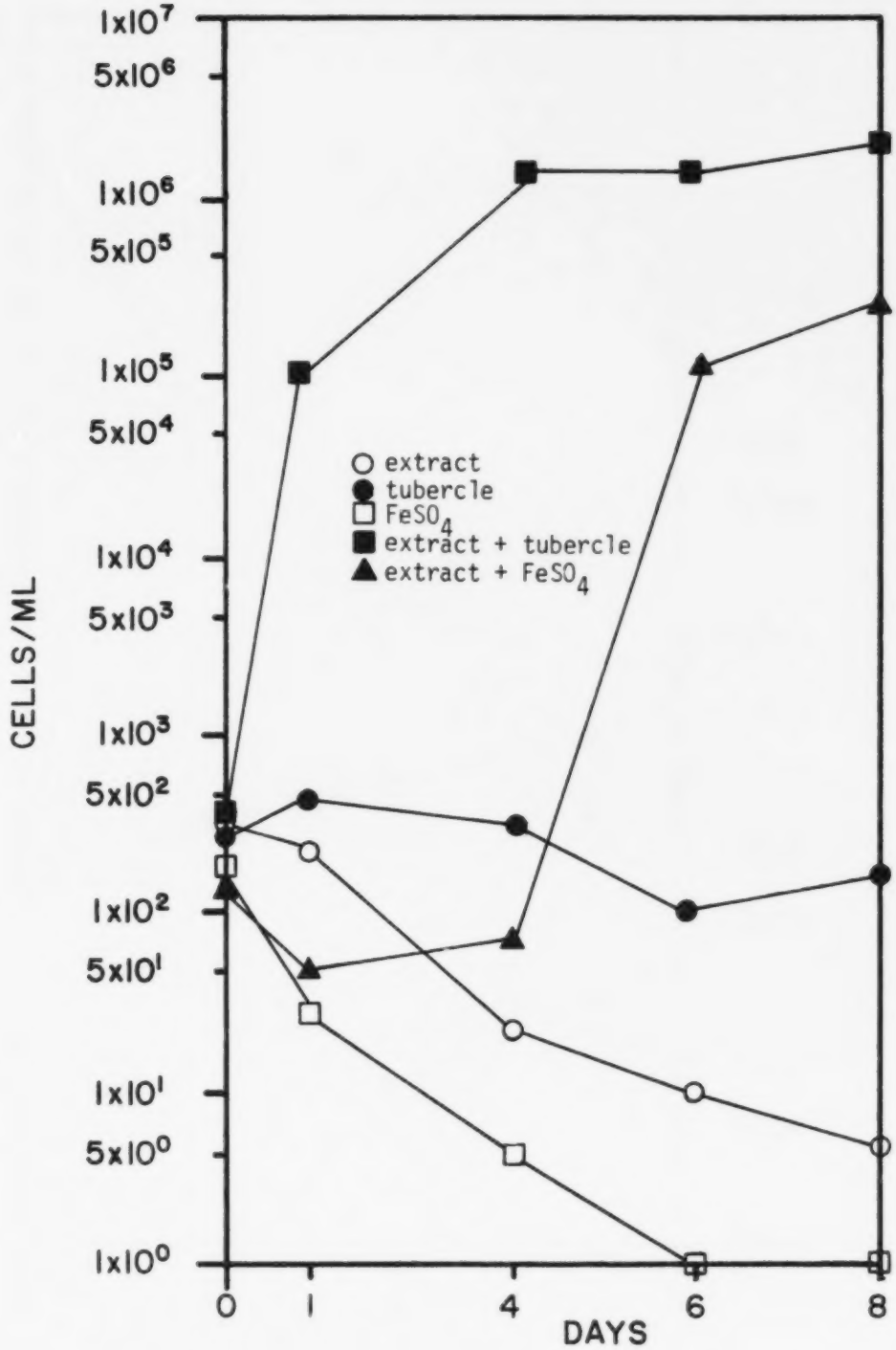

Figure 27. Growth of Arthrobacter sp. in various media.

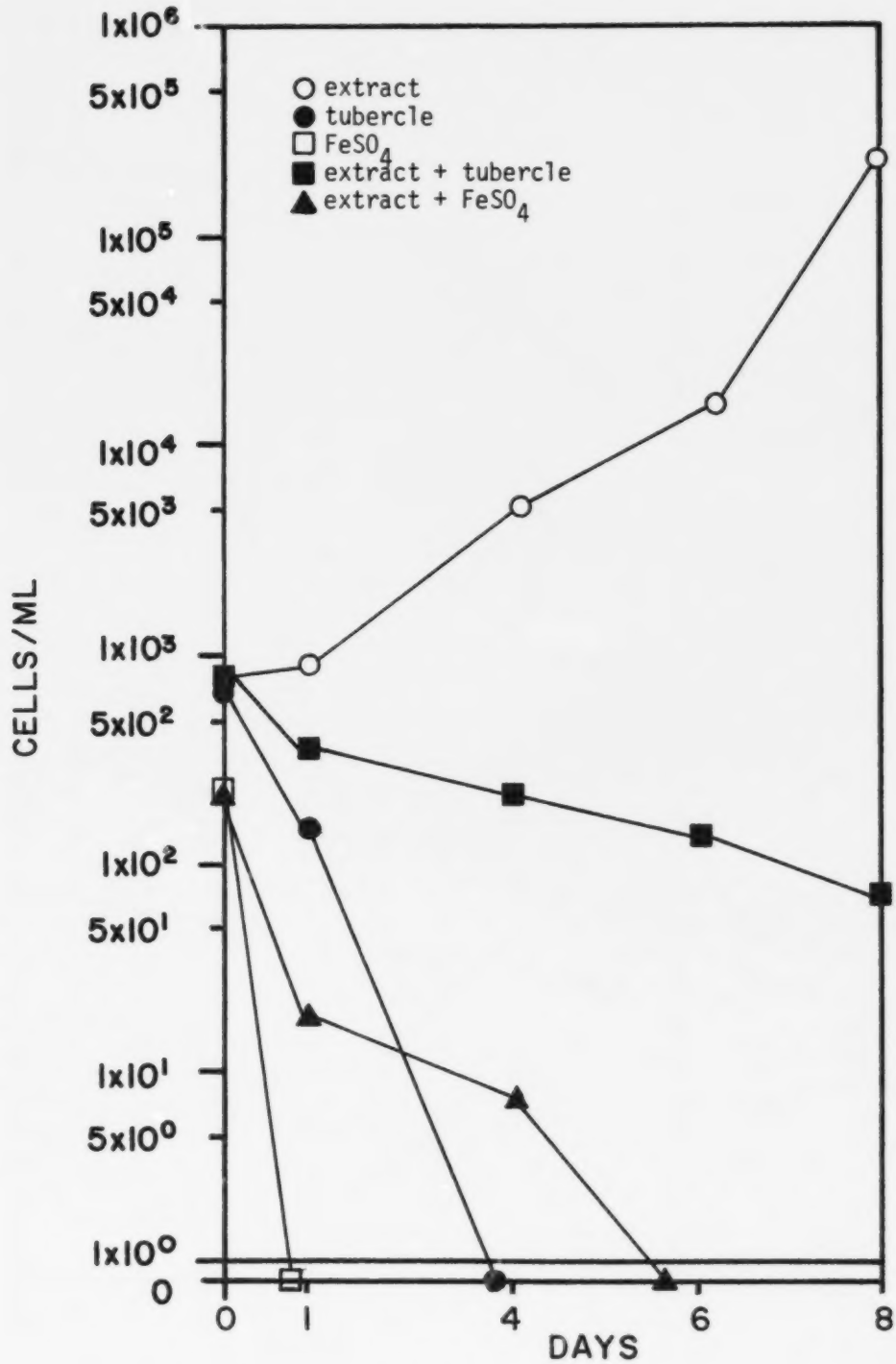

Figure 28. Growth of E. coli in various media.

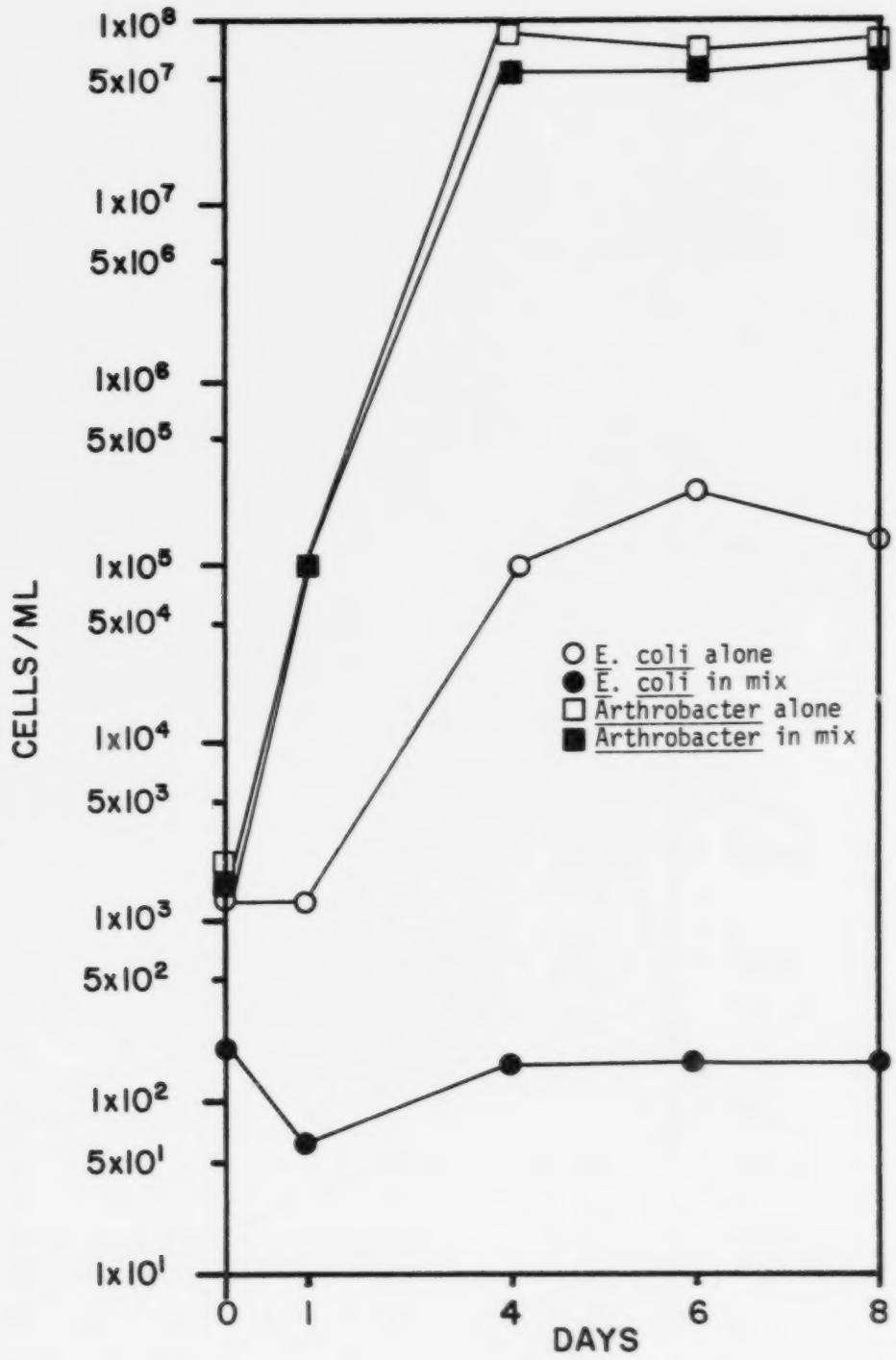

Figure 29. E. coli and Arthrobacter sp. (1:10) in bacterial extract.

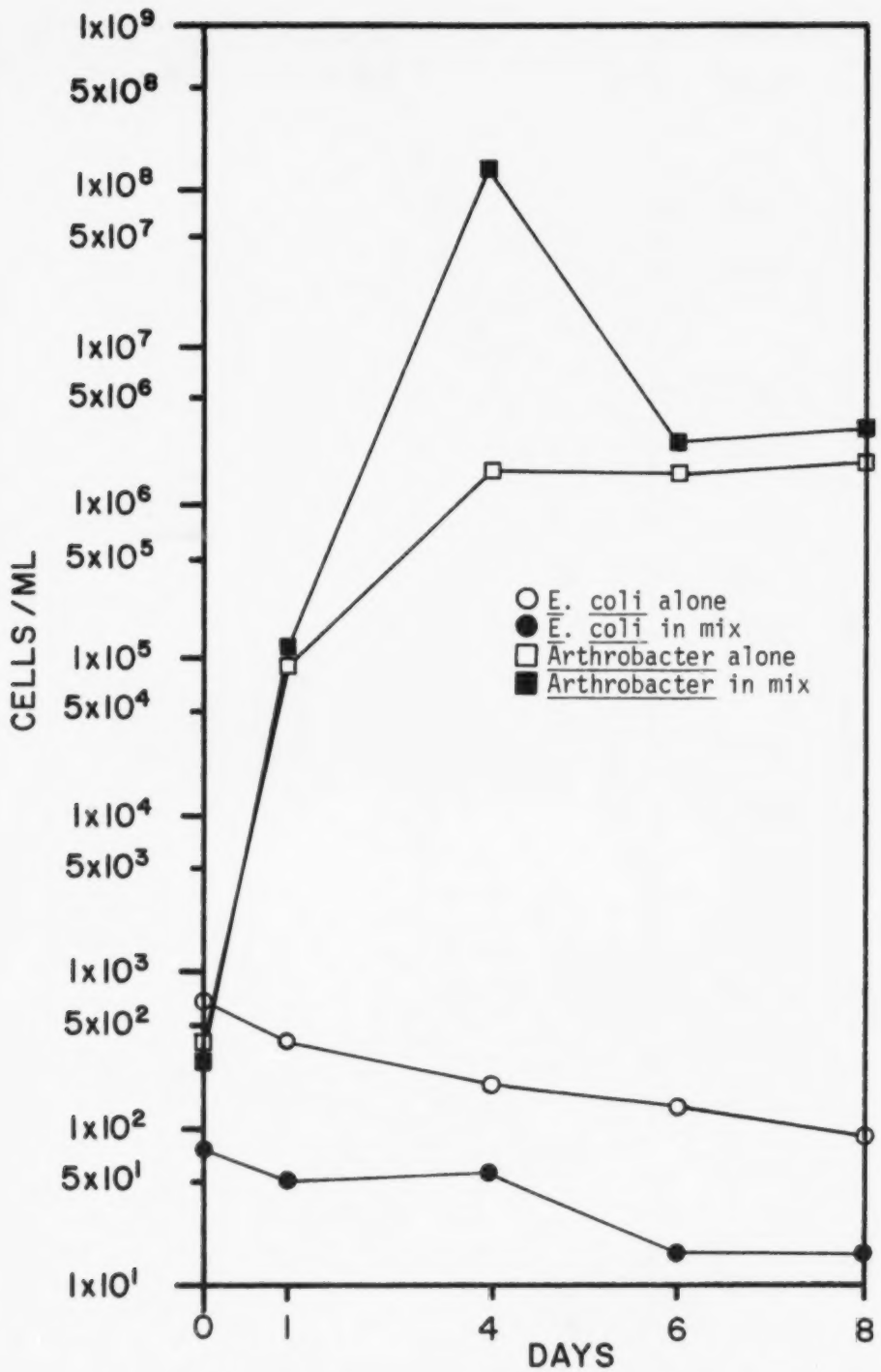

Figure 30. E. coli and Arthrobacter sp. (1:10) in bacterial extract supplemented with crushed tubercle solids.

The Effect of the Physiological Status of Either Member of an Interaction--

In previous experiments where the interaction of E. coli and Flavobacterium was studied, we observed inhibition of the coliform by the non-coliform when the two were inoculated together into non-coliform extract (Figures 1-6). In these experiments the numbers of the Flavobacterium controls increased with time reaching levels of 10^5 - 10^6. However, when the non-coliform is physiologically changed as is indicated by the control not growing and increasing in numbers in the extract, the ability of it to inhibit the coliform is altered. In figures 31 and 32 it can be seen that when the Flavobacterium control is only able to maintain itself in the medium and the coliform control is increasing, the interaction of the two organisms is affected. When the two organisms are grown together in the same medium the coliform is not inhibited. The Flavobacterium is unable to inhibit the coliform and is unaffected by the presence of the coliform.

The physiological state of the coliform member of the interaction is also important in determining the outcome of an interaction. When E. coli and Arthrobacter were grown on non-coliform extract at 6.6° C, the coliform control (Figures 33-34) maintained itself over the eight day course of the experiment. An additional assay at eighteen days indicated only a slight decrease in viable numbers. The Arthrobacter control only increased approximately twenty fold over the course of the eight days (Figure 33). When the two were grown together in a 1:1 ratio in non-coliform extract at the low temperature (Figure 34) Arthrobacter numbers increased over the control reaching a final value of 10^6 organisms/ml whereas the coliform numbers remained unchanged. Under these conditions the coliforms were not inhibited. Recovery of the coliforms was done using PCA and Endo pour and spread plates. The use of the selective Endo medium and the pour plate which subjects the organisms to a high temperature stress might be expected to recover less organisms if the coliforms in the mix were stressed by the presence of the non-coliforms. This does not appear to be the case. Coliform numbers were similar to those observed for the control. Recovery on PCA and Endo pour and spread plates was similar. It appears then that the coliforms may be less vulnerable to inhibition or stress by non-coliforms when either member of the interaction is not actively growing.

TURBIDITY EXPERIMENTS

The purpose of this series of experiments was to determine if non-bacterial turbidity such as that due to natural minerals, hydrated oxides and organic debris was responsible for inhibition of coliform recovery. The study was carried out at the Wilmington Water Department, Wilmington, Delaware using various types of water and turbidity. The turbidity was either added to the water, in turbidity augmentation studies, or removed from the water, in turbidity reduction studies. Since this series of experiments was concerned with the effect of non-bacterial turbidity on coliform recovery an attempt was made to alter the turbidity of the water samples without altering the total bacterial count. This was done in the turbidity augmentation studies by adding turbidity which was

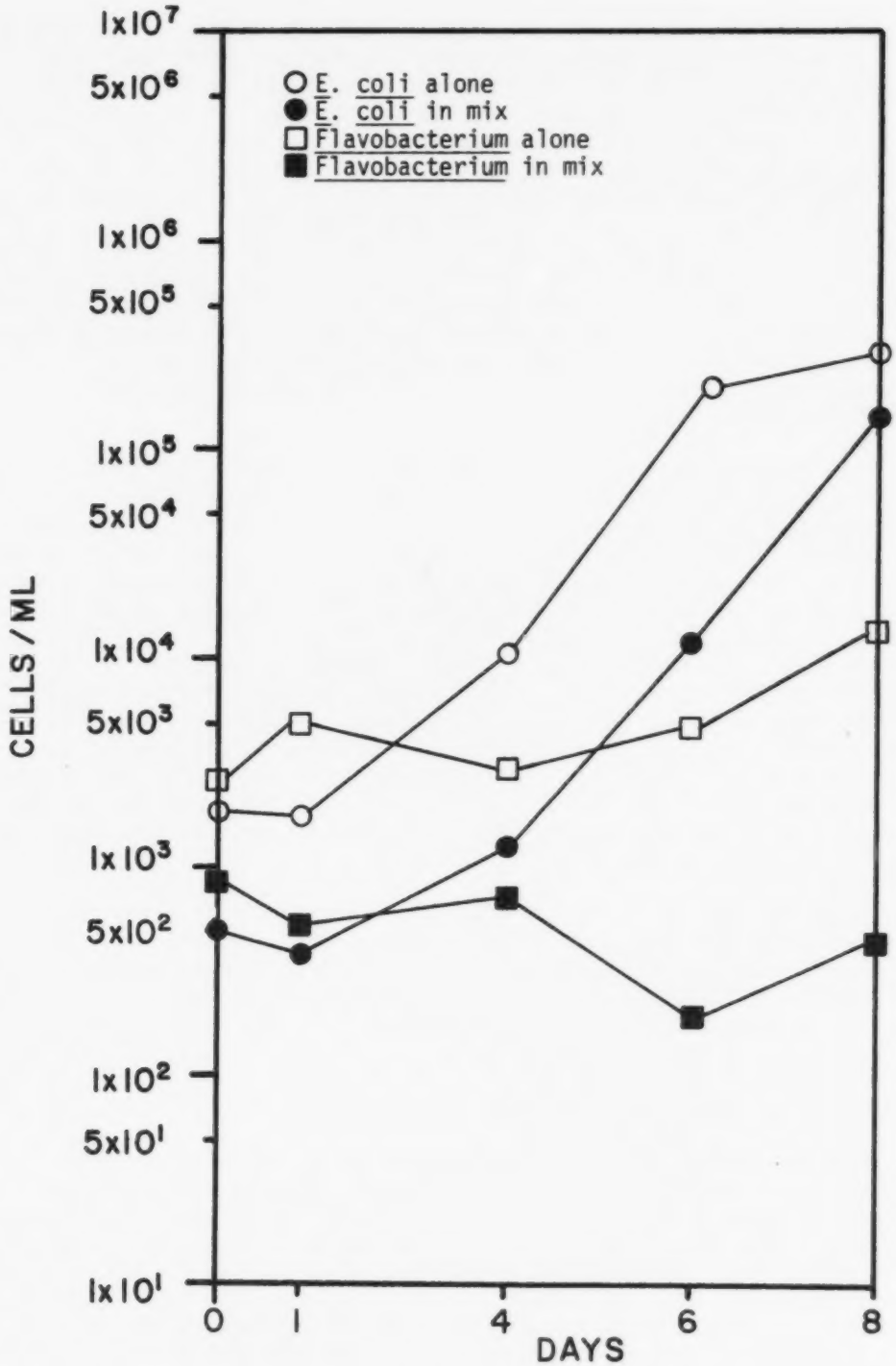

Figure 31. E. coli and Flavobacterium sp. (1:1) in bacterial extract.

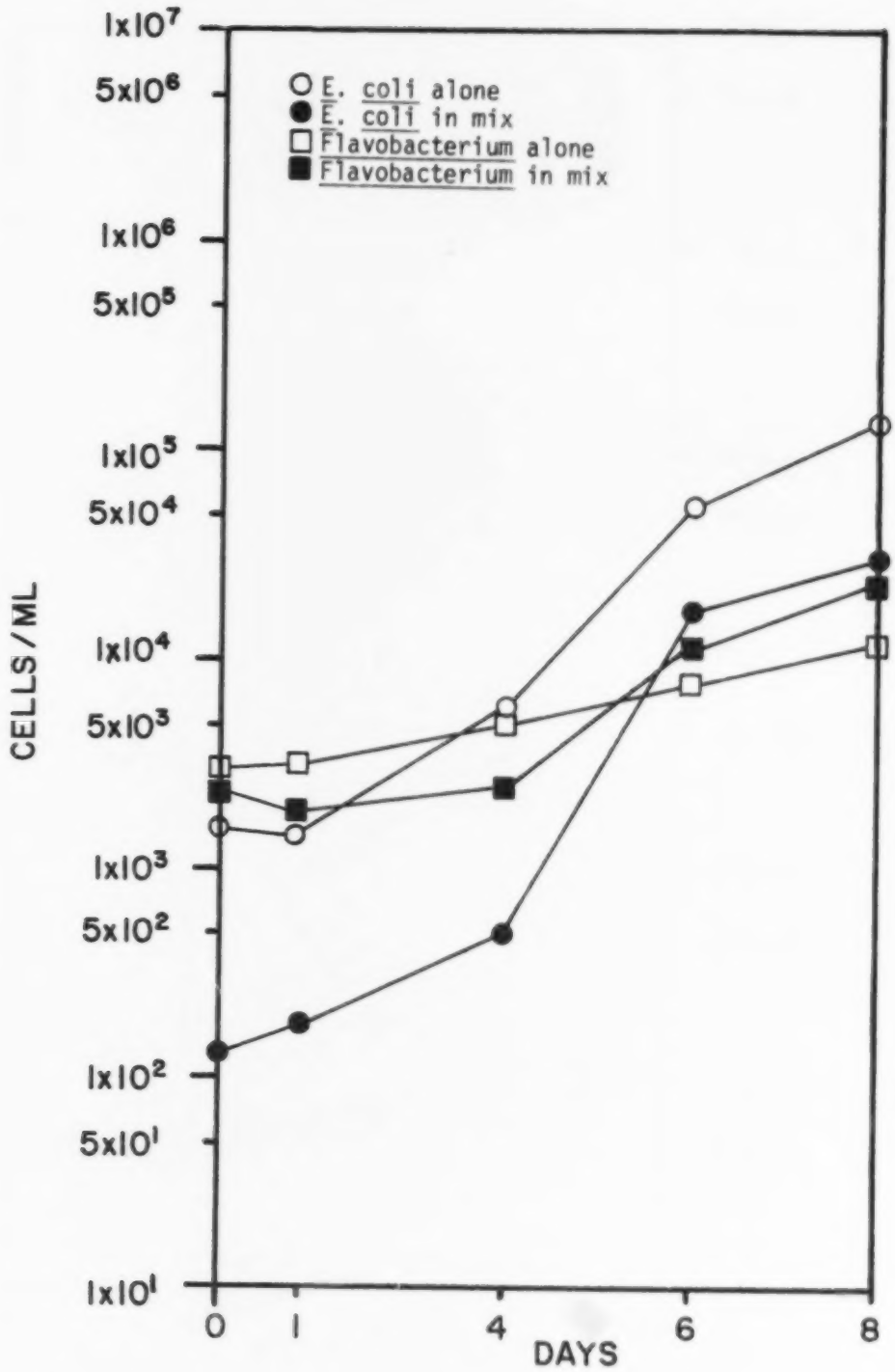

Figure 32. E. coli and Flavobacterium sp. (1:10) in bacterial extract.

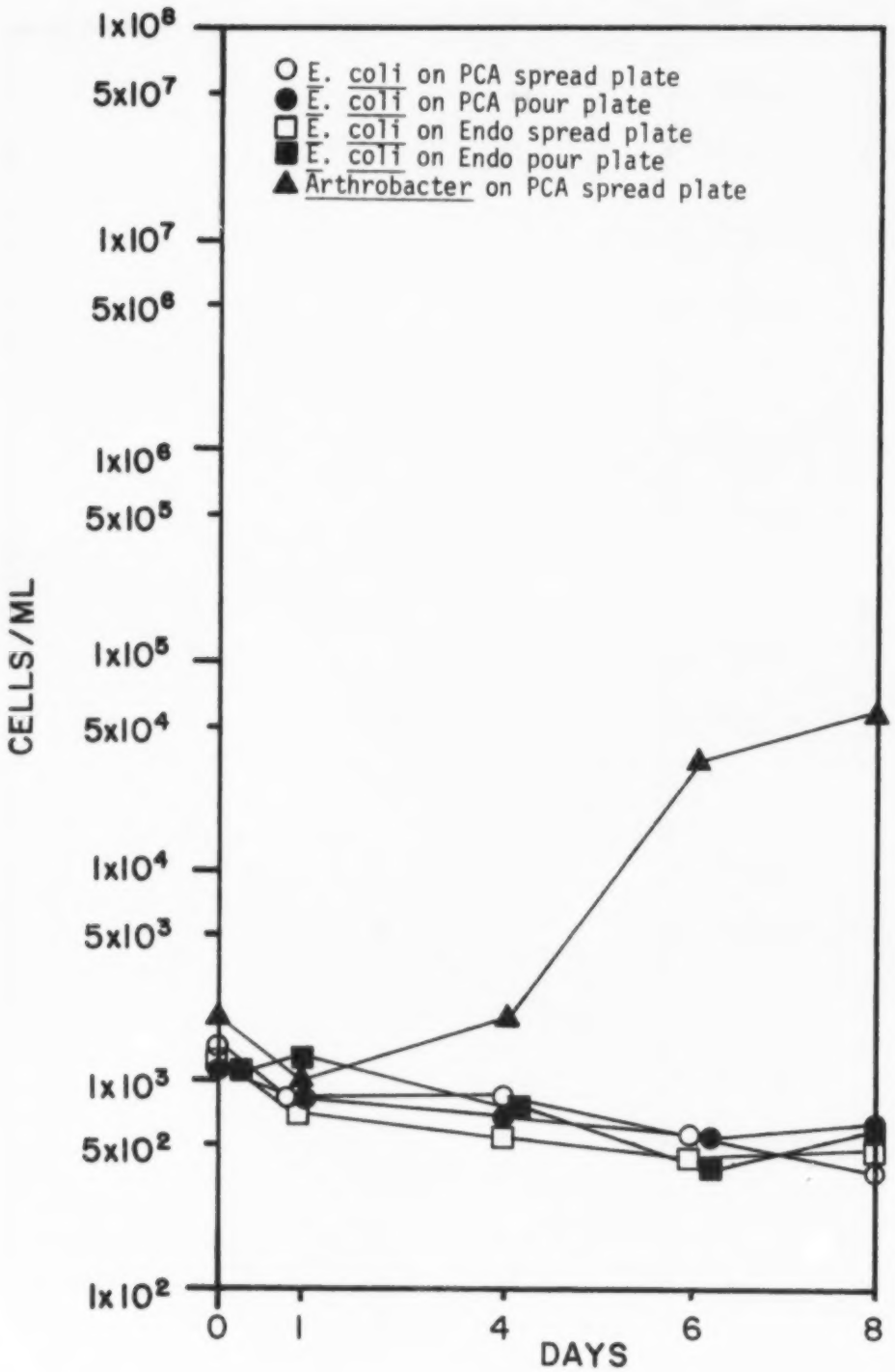

Figure 33. Growth of E. coli and Arthrobacter sp. in bacterial extract at 6.6°C.

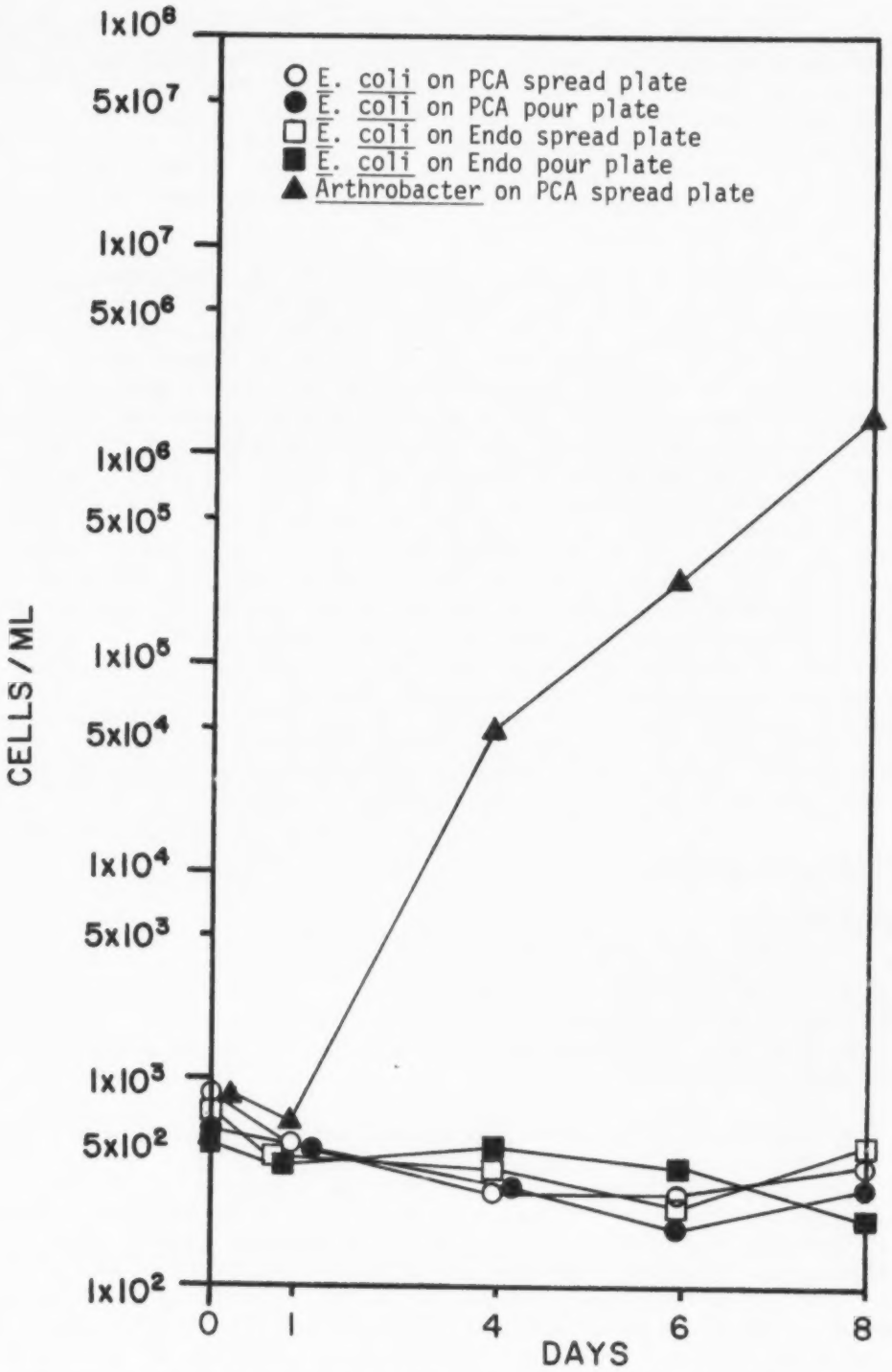

Figure 34. E. coli and Arthrobacter sp. (1:1) in bacterial extract at 6.6°C.

assayed and known to be bacterial free and in the turbidity reduction studies by using very low speed centrifugation which would not pellet bacteria. As a result of these manipulations the resulting two water samples would vary only in the level of non-bacterial turbidity. If this non-bacterial turbidity was the major factor in inhibition of coliform recovery then the water containing the higher level of non-bacterial turbidity would be expected to yield fewer coliforms than the low turbidity water.

Since this was not observed, additional studies were done with some of the water samples to further distinguish the role of bacterial and non-bacterial turbidity in inhibition of coliform recovery. As in the first series the two samples differed in levels of non-bacterial turbidity, but had similar numbers of bacteria. From these samples increasing volumes of water were removed and membrane filtered. In the filtration series for both of the two waters inhibition of coliform recovery was observed to be a function of the volume filtered with the greatest inhibition observed for the highest volume filtered. Since the two original water samples varied only in their levels of non-bacterial turbidity, filtering the same volume from each original water sample resulted in the deposition on the filter pad of the same number of bacteria but different levels of non-bacterial turbidity. As was noted, the high volume sample from each of the two waters showed the greatest inhibition of coliform recovery. If the coliform recovery of these two samples was comparable, it would appear that the inhibition is due primarily to high levels of bacteria. If however coliform recovery was inhibited to a greater extent in the high volume sample from the original high turbidity water, then inhibition would appear to be due primarily to the presence of high levels of non-bacterial turbidity.

Materials and Methods

Water Samples--

The waters used in the turbidity experiments were samples of raw water, distribution water and recirculating water from a main excised from an area of the distribution system which had regrowth problems. The excised main was two meters long and had a 150 mm diameter. Feed water for the main came from areas of the distribution system which had lost their chlorine residual.

In some early experiments raw water was used because it guaranteed the presence of coliforms particularly during the winter months. Water for later experiments was usually taken from remote areas of the Wilmington distribution system carrying little or no chlorine. These locations also had a history of coliform occurrence ranging from occasional to frequent. Because the actual coliform level was difficult to predict, an attempt was made to maximize coliforms by blending samples from two or more "trouble spots," and by occasionally adding small amounts of raw water or water from the isolated main. Despite these efforts many membrane filters had low colony counts, a situation familiar to those who routinely search for coliforms in distribution systems.

Turbidity Sources--

In mains known to be carrying chlorine, but where loose deposits were also suspected, a surging of the main frequently yielded a moderate chlorine, high turbidity mixture at hydrants. When such suspensions were collected in sterile containers without thiosulphate and stored at 2 - 4°C for several weeks many of them were then found to be chlorine-free and bacteria-free.

Confluence situations also yielded suspensions suitable for turbidity studies. When water traveling by one route degrades to a turbid, chlorineless condition, and then converges with water which has traveled by a superior route, retaining its chlorine, this mixture can often be refrigerated for a few weeks to yield an apparently sterile chlorine-free turbidity.

Turbidity Measurement and Centrifugation--

Centrifugal separation of bacteria from other turbidity was accomplished in an International UV Centrifuge using 50 ml sterile capped tubes. G forces used were from 160 to 450 and were maintained for 6 or 8 minutes. The decanted supernatants were pooled before use.

Turbidity was measured using a DRT-100 nephelometer type instrument.

Coliform Recovery--

Coliform recovery was assayed by both MPN and MF in case one of these techniques was more sensitive to inhibition by turbidity. It is difficult to compare the absolute numbers obtained using these two techniques because the MPN number actually represents a range. It was therefore necessary to determine if the MF coliform number fell within the MPN range.

MPN coliform measurements used Lauryl Sulphate Broth (sometimes called Lauryl Tryptose Broth), 20 ml of 1 1/2 strength broth for 10 ml portions, and single strength for 1 ml and smaller portions. 3 or 4 groups of 3 decimal ranks of 5 tubes each were employed and presumptive positives were transferred to Brilliant Green Bile Broth for confirmation.

Membrane filter measurements of coliforms were made with both LES Endo agar and MF Endo broth usually without enrichment. The broth was used more often on the assumption that more routine analyses in the water industry are being conducted on this medium. Incubation was for 22 to 24 hours at 35°C. Unless indicated in the text, MF coliforms were not confirmed. Therefore the numbers reported as MF coliforms represent the highest possible number of these organisms recovered. Even with this bias it was observed that in many experiments increasing the volume of the water sample filtered resulted in decreased recovery of coliforms on a per ml or 100 ml basis. Therefore of even greater interest than confirmation of colonies which looked like coliforms is the analysis of colonies which do not show up as coliforms. This is in principle technically possible, but in practice not feasible. However, to determine if perhaps there were coliforms present on the MF filters which were not growing into colonies, circular 50 mm^2 discs were cut from inoculated membrane filters with a

sterile cork borer and transferred to Lauryl Sulphate Broth and confirmed in Brilliant Green Bile broth.

Total Bacterial Recovery--

Measurement of the total bacterial population was done using M-5 agar (3 grams Plate Count Agar, 12 grams plain agar, 200 mg ferrous gluconate in 1000 ml distilled water) formulated by H. T. Victoreen. The suitability of this agar for this purpose and its superiority to plate count agar is evident from the data in Table 4 where pour plate results with Plate Count Agar (Standard Methods Agar) are compared with M-5 agar using a variety of water samples over a 7 month period. Both inoculated agars were incubated for 7 days at 28°C.

TABLE 4. A COMPARISON OF BACTERIAL RECOVERY ON PLATE COUNT AGAR AND M-5 AGAR

Source	Date	PC	M-5	M-5/PC
Grove. Pr.	3-21-77	4	48	12
K. Butler	3-21-77	13	180	14
ch Lore	3-21-77	340	6000	18
Wynd.	3-23-77	4	22	5.5
Br. Ns.	3-23-77	320	2700	8.4
Pr. Ess.	3-25-77	1300	3200	2.5
708 Pr.	3-25-77	0	150	
Wynd	4- 3-77	2	100	50
N.P.	4-13-77	140	2000	14
Br. Ns.	4-13-77	30	1500	50
Ch. Lore	4-29-77	7000	13000	1.9
Ch. Lore	5- 6-77	1600	5000	3.1
Woodcr.	5- 6-77	6	36	6
Gov. Pr.	5- 9-77	28	240	8.6
Br. Ns	6-22-77	450	5600	12
Br. Ns	7- 6-77	740	14000	19
1509	9- 7-77	46	6500	140
K. Butler	9-26-77	180	2200	12
Eastm.	9-26-77	76	2500	33
1212	9-28-77	40	2700	68
Han. Wash	10-26-77	90	6200	69
Lower Garl	10-26-77	54	4800	89
Maria	11- 7-77	100	8000	0.80

Studies Using Raw Water

A group of preliminary experiments were done on membrane filters to determine whether in a turbid sample coliform yields would be obtained which were proportionate to sample size. In these experiments raw water of good quality was used. The water samples had phosphate levels of less than 0.4 and ranged in turbidity from 2.0 to 10.0 FTU. The results appear in Table 5.

TABLE 5. COLIFORM RECOVERY FROM GOOD QUALITY RAW WATER

Date	Source	Sample Size (ml)	Colonies Counted	Calculated Coliforms/100ml
9/29/77	Raw Porter*	0.5	19	$3.8X10^3$
		2	25	$1.3X10^3$
	Raw Brandywine*	0.5	28	$5.6X10^3$
		2	51	$2.6X10^3$
10/10/77	Raw Brandywine	0.1	36	$3.6X10^4$
		0.2	55	$2.8X10^4$
		0.5	90	$1.8X10^4$
10/16/77	Raw Brandywine*	1	2	200
		2	7	350
		4	6	150
		4	7	175
		8	16	200
		50	71	140
		100	80	80
		150	75	50

*LES Endo agar with enrichment. On remaining dates the same medium was used without enrichment.

It is apparent that despite variations in source, turbidity and coliform density, smaller volumes filtered gave better calculated yields on the same sample. When a smaller sample volume is filtered, less turbidity and bacteria are being spread over the effective filtering area. Increasing the sample size filtered may result in inhibition of coliform recovery by turbidity, non-coliforms, coliforms or an undefined crowding factor. Microbial inhibition may be a result of toxic by-products of other organisms or competition for space and nutrients.

Another experiment was conducted with an intermediate quality raw

water centrifuged at 200g for 6 minutes to reduce the turbidity from 18 to 3.0. Coliform recovery was determined using Endo broth without enrichment. Results appear in Table 6.

TABLE 6. COLIFORM RECOVERY FROM INTERMEDIATE QUALITY RAW WATER

Quantity Filtered	Pre-Centrifugation (Turbidity = 18) Coliforms/100 ml	Post-Centrifugation (Turbidity = 3) Coliforms/100 ml
2-0.05 ml portions	6.1×10^4	6.4×10^4
4-0.20 ml portions	2.88×10^4	2.85×10^4
3-0.50 ml portions	1.73×10^4	1.83×10^4

Total bacterial numbers were determined using M-5 agar incubated for 7 days at 28° C. Reduction of turbidity from 18 to 3 FTU did not reduce total bacterial numbers as evidenced by the same bacterial counts (1.25×10^5 organisms/ml) obtained on M-5 agar before and after centrifugation. In addition, the pattern of decreasing yield with increasing sample size was again observed for the pre- and post-centrifuged samples. Since comparable numbers of coliforms were recovered from a given volume of each of the two water samples, it would appear that turbidity *per se* is not responsible for the decreased coliform yield obtained when larger volumes of water are filtered. The observed pattern may therefore be due to the continued crowding of colonies on the filter surface with a resultant competition for space and nutrients or from the by-products of the competing organisms.

Studies Using Distribution Water

Distribution water was used to study the effect of turbidity augmentation on coliform recovery. The water used was a composite made of water from five areas, each with a history of coliform regrowth. 2% raw water was then added to this mix to further improve the chances of detecting coliforms during the experiment. All of the five finished water components had lost their chlorine in the distribution system.

The mixture was divided in half and one half was augmented with additional discolored water deposits to raise the turbidity from 1.2 to 6.6. These deposits were obtained by collecting from an area containing chlorine in a sterile thiosulphate free container and refrigerating for several weeks until the deposit was chlorine free.

Coliform determinations were made on the water before and after turbidity augmentation by the MPN and membrane filter techniques. Results are reported in Table 7.

TABLE 7. COLIFORM RECOVERY FROM DISTRIBUTION WATER

	Pre-Augmentation (Turbidity = 1.2)	Post-Augmentation (Turbidity = 6.6)
Confirmed MPN*	49/100 ml	40/100 ml
MF Counts 6 - 10 ml portions	15/100 ml	15/100 ml
6 - 50 ml portions	21/100 ml	19/100 ml

*reported figures represent an average of 4 sets of 15 tubes MPNS.

Parallel assessments of the overall bacterial population were made by 7 day plate counts at 28° C on M-5 agar. The M-5 counts were 2.5×10^3 organisms/ml for the pre-augmentation water and 2.6×10^3 organisms/ml for the post-augmentation water indicating that the added turbidity concentrate was bacteria-free and that the only change it brought about in the coliforms' environment was to raise the amount of suspended matter. Under such conditions, there is no impairment of coliform recovery by either method, attributable to the turbidity augmentation. In addition, filtering a five fold greater portion through the millipore filter (50 ml as opposed to 10 ml portions) did not result in a proportionate loss of coliform recovery. However, when 4 50 mm^2 discs were removed from 2 sets of pre- and post-augmentation filters in each case one of the 4 filters was positive in L.S. broth. This may indicate that in both cases there were inhibited or stressed coliforms on the filter which would not have been considered as such if special efforts for their recovery had not been undertaken. In this composite water coliforms are outnumbered by the non-coliforms by a factor of 5×10^3.

In another experiment with distribution water the water temperatures were so low that there was no assured source of coliform bearing water in the distribution system. It was therefore necessary to take a stock coliform culture previously isolated from a coliform regrowth area, dilute it heavily, and adapt it to a medium consisting of membrane filtered water from this same location fortified with pulverized, sterilized main tubercles. This produced a suspension which 48 hours later had a coliform MPN of 490. When this suspension was riled and allowed to settle for 30 minutes, the supernatant had an MPN of 130. This supernatant was employed in the experiment discussed below.

A freshly drawn 50 - 50 mix from two areas showing coliforms during warmer weather was divided into two sterile flasks, brought up to room temperature and inoculated with a 10% addition of the supernatant described above. This gave a turbidity of 5.6. One of these flasks received a further addition, an amount of bacteria-free, chlorine-free, main deposits sufficient to further raise the turbidity to 11.4. After 30

minutes contact coliform and M-5 plate counts were determined on both flasks. Coliform recovery is reported in Table 8.

TABLE 8. COLIFORM RECOVERY FROM DISTRIBUTION WATER

	Pre-Augmentation (Turbidity = 5.6)	Post-Augmentation (Turbidity = 11.4)
Confirmed MPN*	7.7/100 ml	4.0/100 ml
MF Counts	1.6/100 ml	2.8/100 ml

*Reported figures represent an average of 4 sets of 15 tube MPNs.

M-5 agar counts were 1.6×10^4 organisms/ml for the pre-augmentation water and 1.7×10^4 organisms/ml for the post-augmentation water, again indicating that the added turbidity did not contain viable bacteria. When the MF and MPN results are compared the MF results do fall into the 95% confidence limits of the MPN. The original water, however, had an MPN of 130 (range = 30 - 3100 coliforms/100 ml) before being diluted 1:10. The data is suggestive here, then, that recovery by either method may be at the lower end of the expected range. This may mean that factors such as the high numbers of non-coliforms which outnumber the coliforms by a factor of 10^6 or the turbidity present in the 5.6 FTU water is high enough to inhibit coliforms or coliform recovery and increasing the turbidity to 11.4 does not further contribute to inhibition of coliform recovery.

Studies Using Recirculating Distribution Water from an Excised Main

The main used, as was previously noted, was excised from an area of the distribution system which had regrowth problems. A study was done using distribution water which had recirculated through this main by blending unfiltered water from the main with some which had been fiber glass filtered. This resulted in a discolored water sample with a turbidity of 8.0. As a precaution against an unexpected drop in the coliform population this mixture was then augmented with low levels of raw water. Coliform determinations were made on this water by both the MPN and MF methods and the total bacterial population was evaluated using M-5 agar with 7 days incubation at 28°C. The water was then centrifuged at 460 x g for 7 minutes and the entire evaluation repeated. The final turbidity was 1.3. The coliform data is reported in Table 9.

TABLE 9. COLIFORM RECOVERY FROM RECIRCULATING MAIN WATER

	Pre-Centrifugation (Turbidity = 8.0)	Post-Centrifugation (Turbidity = 1.3)
MPN*		
1st set	230/100 ml	330/100 ml
2nd set	330/100 ml	330/100 ml

(Continued)

TABLE 9. (Continued)

	Pre-Centrifugation (Turbidity = 8.0)	Post-Centrifugation (Turbidity = 1.3)
MF Counts		
1 ml portion	1400/100 ml	800/100 ml
2 ml portion	950/100 ml	500/100 ml
4 ml portion	180/100 ml	
6 ml portion		180/100 ml
8 ml portion		75/100 ml

*Reported figures represent an average of 4 sets of 15 tube MPNS.

M-5 counts indicated a decline in bacterial numbers from an average of 6.9 x 10^5 organisms/ml in the original 8 FTU water to 5.7 x 10^5 organisms/ml in the 1.3 FTU water. This reduction in total bacterial numbers is also reflected in a reduction of coliform recovery by the MF technique. However, the MF results are observed to vary dramatically as a function of the volume filtered. The phenomenon is observed with both the pre- and post-centrifuged waters. The pre-centrifuged water has an approximately seven fold higher level of turbidity. If turbidity were primarily responsible for inhibition of coliform recovery, the recovery of organisms from the low turbidity water would have been greater than that which was observed. Centrifugation removed 18% of the total organisms, and therefore presumably also of coliforms. The coliform numbers recovered in the post-centrifuged water reflect at least this 18% decrease. Therefore it appears that removal of turbidity did not enhance coliform recovery.

A later coliform recovery experiment was done using 80% of the water from the recirculating main and 20% from a high rust distribution area. This mixture had a turbidity of 15.5. After 7 minutes centrifugation at 1600 RPM the turbidity was reduced to 2.6. Coliform recovery before and after centrifugation using the MF and MPN procedures is presented in Table 10.

TABLE 10. COLIFORM RECOVERY FROM A COMBINATION OF DISTRIBUTION WATER AND RECIRCULATING MAIN WATER

	Pre-Centrifugation (Turbidity = 15.5)	Post-Centrifugation (Turbidity = 2.6)
Confirmed MPN*	720 /100 ml	490 /100 ml
MF Counts		
5 - 1.0 ml volumes	180/100ml	220/100ml

*Reported figures represent an average of 3 sets of 15 tube MPNS.

The rusty deposits which constituted most of the turbidity present were easy to remove and the centrifugation procedure removed approximately 83% of the turbidity without effectively altering the bacterial levels present. Under these conditions coliform recovery did not appear to be effectively changed as a function of turbidity removal. M-5 counts (7 day incubation at 28^0C) were an average of 1.4×10^6 organisms/ml for the pre-centrifuged water and 1.2×10^6 organisms/ml for the post-centrifugaed water. Centrifugation therefore removed 83% of the turbidity and 14% of the total bacterial numbers. If turbidity per se were inhibiting coliform recovery both the MF and MPN techniques might be expected to indicate improved recovery of coliforms. This does not appear to be the case.

Another coliform recovery experiment was done using 23% of the water from the recirculating main, 73% of the water from the distribution system and 2% rusty water from the distribution system. The mixture had a turbidity of 5.1 and was centrifuged for 7 minutes at 1650 RPM to give a turbidity of 1.1. Coliform recovery before and after centrifugation using the MF and MPN procedures is presented in Table 11.

TABLE 11. COLIFORM RECOVERY FROM A COMBINATION OF DISTRIBUTION WATER AND RECIRCULATING MAIN WATER

	Pre-Centrifugation (Turbidity = 5.5)	Post-Centrifugation (Turbidity = 2.6)
Confirmed MPN*	530/100 ml	260/100 ml
MF Counts		
4 - 5 ml portions	25/100 ml	30/100 ml
4 - 25 ml portions	37/100 ml	26/100 ml

*Reported figures represent an average of 3 sets of 15 tube MPNs.

M-5 counts (7 day incubation at 28^0 C) were an average of 5.0×10^5 organisms/ml for the pre-centrifuged water and 3.3×10^5 for the post-centrifuged water. Centrifugation therefore removed 78% of the turbidity and 33% of total organisms.

In this experiment the MF counts are lower than the range obtained using the MPN. In addition the MPN numbers obtained after centrifugation of the water reflect a loss of organisms, whereas the numbers obtained using the MF technique do not. Using the MF technique, filtering 5 or 25 ml portions yielded a similar number of colonies on the filter. One ml portions had also been run, but the numbers were so low as to be statistically unreliable. It may be for the levels of turbidity and organisms present in the water that larger volumes would have had to be used to see inhibition of coliform recovery as a function of the volume filtered.

Two additional types of manipulations were done in this experiment.

Discs of 50 mm^2 size were aseptically removed from the apparently barren intercolonial spaces on certain membrane filters after 24 hours incubation and transferred to single strength Lauryl Sulphate Broth. In the case of the pre-centrifuge filters 2 discs were cut from each of 2 - 5.0 ml filters and 4 discs from a 25 ml filter. In the case of the post-centrifuge filters 2 discs from each of 2 - 5.0 ml filters and 2 discs were cut from each of 2 - 25 ml filters. All 16 of these discs produced gas in Lauryl Sulphate Broth in 18 hours and confirmed in Brilliant Green Bile. To say that turbidity was not restricting recovery is not to say that all the coliforms were detected. The fact that all of the 50 mm^2 discs from apparently barren areas of both pre- and post-centrifuge filters were positive in Lauryl Sulphate Broth and confirmed in Brilliant Green Bile suggests heavy microbial interference. Apparently, coliforms which had not grown into recognizable colonies were trapped under the surface mat on the filters and removal of 78% of the turbidity left the mat just as restrictive as before. There was also imperfect coliform detection among the MPNs. When one 0.1 ml set from the pre-centrifugation group and one 0.1 ml set from the post centrifugation were all transferred to Brilliant Green Bile, one more tube showed positive in each set. The sets chosen were among those which had displayed a mixture of positive and negative tubes. If the effort to find coliforms among the negative, but turbid Lauryl Sulphate tubes had been extended to all sets, it is possible that more would have been found. Such studies have previously been done at the Wilmington Water Department (13). In these studies transfers were made from negative but turbid Lauryl Sulphate Broth tubes into Brilliant Green Bile broth tubes. The Brilliant Green Bile broth tubes gave positive results, and further examination of the organisms in these tubes indicated that coliforms were present. This study along with the ones presented in this report indicate that numerous factors influence coliform recovery. This should be taken into consideration in the interpretation of microbiological data obtained from assaying distribution system water.

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